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Mechanisms of Condition-Specific Regulation of mRNA Stability by Puf Proteins: From Yeast to Humans

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B.S., Biology, University of Missouri-Saint Louis, 2008

Submitted to the Graduate School at the University of Missouri-Saint Louis in Partial
Fulfillment of the Requirements for the Degree

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With an Emphasis in Cell and Molecular Biology

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ABSTRACT

Regulation of gene expression is critical to properly functioning cells. In addition to the well understood transcriptional control, post transcriptional control of gene expression expands our understanding of a multifaceted system that allows for precise protein production. One such mechanism of post transcriptional control of gene expression is the regulation of mRNA decay and translation rate facilitated by RNA binding proteins. Controlling mRNA metabolism allows the cell to make rapid changes in expression patterns by utilizing already available mRNA, bypassing the wait for newly transcribed mRNA. The Puf family of RNA binding proteins bind specific mRNAs through interactions with cis-regulatory elements located in the 3' untranslated region (UTR) consisting of a core UGU sequence. Puf proteins are conserved throughout eukaryotes and have diverse roles including stem cell maintenance, neuronal development, stress response and organelle biogenesis. Concomitant with their cellular roles, Puf proteins bind a variety of target mRNAs containing divergent binding sequences downstream of the core UGU. This work focuses on the recent discovery of Puf proteins role in stress response. In *Saccharomyces cerevisiae*, Puf proteins have been shown to be conditionally active in response to the metabolic state of the cell. Specifically, in fermentative growth Puf proteins stimulate decay of target mRNAs; however, during respiration, Puf proteins are inactive resulting in the stabilization of target mRNAs. Presented herein, I show that in addition to Puf3p, the activity of Puf1p, Puf4p and Puf5p in yeast is conditionally regulated. I establish *YHB1* mRNA as a bona fide target of Puf1p, Puf4p and Puf5p regulation through one unique binding site

located in its 3' UTR. *YHB1* encodes the only known defense gene against nitric oxide stress in yeast. Growth studies show that Puf proteins regulate *YHB1* mRNA in response to nitric oxide stress allowing for an adaptive response. This establishes a model for Puf protein regulation of target mRNAs where transcripts are rapidly turned over in the absence of stress; however, in the presence of stress transcripts are stabilized to increase protein production and combat the stress. Much of the mechanistic detail underlying Puf regulation remained to be elucidated. Herein, I show that Puf activity is regulated by at least two different mechanisms. The alteration of Puf activity is not due to increased expression or altered localization. Instead, the RNA binding activity of Puf5p is reduced during inactivating conditions providing a mechanism for inactivation; however, Puf1p, Puf3p and Puf4p are able to bind target mRNAs in both activating and inactivating conditions. Puf proteins require interactions with decay machinery to facilitate mRNA degradation. The Puf3p interaction with Pop2p is perturbed in inactivating conditions and an increase in association with a truncated form of Pop2p is observed. This provides mechanistic insight into how Puf3p is inactivated in stress conditions. Thus I have established two mechanisms of Puf protein inactivation in yeast. It is likely that more mechanisms exist given that no clear evidence for either mechanism previously described was observed for Puf1p or Puf4p. In a third project, I began to evaluate human Puf proteins, also known as Pumilio proteins. I sought to identify natural targets of Pum regulation in humans. Large scale studies suggest physical association of Pum proteins with mRNAs involved in Parkinson's disease (PD). Utilizing bioinformatic approaches as well as experimental approaches I identified SNCA,

LRRK2 and SAT1 as targets of Pum regulation in humans. The implication of this discovery may provide a novel therapeutic approach to PD in the future as SNCA and LRRK2 are the most commonly associated genes with PD.

DEDICATION

I dedicate this Ph.D. dissertation to my wife Kelsey Michelle Russo who has been with me throughout this exciting journey. Without her support and belief in me this would not have been possible. I could not have designed a better person to be a part of my life. You are my other.

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I would also like to thank my advisor Dr. Wendy May Olivas. Without her I would have never accomplished any of this work. From the moment I took her Nucleic acids class and her biotech II class I was enamored with molecular biology. When I came to her for advice about graduate school and how to begin to approach research she kindly offered me a spot in her lab to give research a try. Well, from there the rest is history. I fell in love with the lab and quickly became a Ph.D. student in Dr. Olivas' lab. I want to extend my sincerest gratitude to her for allowing me to work in her lab and for providing me with support, both in the lab and in my personal life. I hope that our relationship continues for years and decades to come.

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talk about the future with and have someone who genuinely cares about your future.

Without my committee this dissertation would not have been possible.

I want to thank Maryann Hempen and Kathy Burney-Miller for all of their assistance and support. They are the glue that holds the biology department together.

Finally I want to thank some people who made all of this work possible. I want to thank Dr. Mike Nichols who provided me with all the knowhow for starting work with human cell lines. The experience he provided me with will be invaluable throughout my career. Also I must extend my thanks to Roy Parker, Marv Wickens and Aaron Goldstrohm for strains and plasmids that made much of this work possible.

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CHAPTER I: INTRODUCTION

Proper gene expression is critical to the survival of cells. Cells must express the precise amount of protein at specific times to respond to various environmental stimuli. Much research has focused on how the cell regulates gene expression using a variety of transcriptional and post-transcriptional mechanisms. Such mechanisms include post-transcriptional control of messenger RNA (mRNA) stability and translational repression. In addition to vast transcriptional control, the cell can use post-transcriptional mechanisms to control protein expression rapidly, in response to stimuli, by modulating its use of already available mRNA. This crucial layer of control allows for “fine-tuning” of protein expression, providing a rapid response to cellular stress and allowing energy conservation in a variety of nutritional states.

Typically, control elements located in the 3' untranslated region (UTR) of mRNAs recruit regulatory proteins or miRNAs that influence mRNA decay and/or translation rates. These RNA binding proteins bind specific sequences and recruit machinery that influences decay/translation. The cis-elements located in the 3' UTR of mRNAs often dictate what family of RNA binding proteins is recruited. Examples of these cis-elements include AU or GU rich elements which recruit specific proteins to mediate stabilization or destabilization of target mRNAs (Adjibade and Mazroui, 2014). Also, in higher eukaryotes, miRNA recruitment to complementary seed regions, located in 3' UTRS facilitate mRNA degradation/translational inhibition through interactions with Ago and GW182 (Adjibade and Mazroui, 2014). Furthermore, many additional families of RNA binding proteins exist to regulate mRNA decay/translation rates for fine-tuning of protein expression.

One important family of 3' UTR regulatory proteins is the Puf family, with multiple members in mammals, insects, worms, plants and yeast. Pumilio from *Drosophila melanogaster* (DmPum) and FBF from *Caenorhabditis elegans* were the founding members of this group providing the Puf family name. DmPum promotes abdominal segmentation in the early embryo by binding the 3' UTR of *hunchback* mRNA and subsequently repressing its translation (Murata and Wharton, 1995) and promoting its deadenylation (Wreden *et al.*, 1997). DmPum also represses translation of *cyclin B* mRNA for regulation of germline stem cell development (Forbes and Lehmann, 1998; Asaoka-Taguchi *et al.*, 1999; Parisi and Lin, 1999) and regulates anterior patterning (Gamberi *et al.*, 2002). The FBF proteins promote the sperm/oocyte switch by binding the 3' UTR of *fem-3* mRNA and repressing its expression (Zhang *et al.*, 1997), and control germline stem cell maintenance by repressing *gld-1* mRNA expression (Crittenden *et al.*, 2002).

Different organisms contain variable numbers of Puf proteins, from one in *D. melanogaster*, to over 20 in *Arabidopsis thaliana* (Barker *et al.*, 1992; Horan *et al.*, 2005). Further examples include *Saccharomyces cerevisiae*, which expresses six Puf proteins and humans which express only two Puf proteins. Concomitant with variable numbers in different organisms, Puf proteins regulate diverse processes including cell development, stem cell maintenance, organelle biogenesis and environment stress defense by binding to specific sequences of targeted mRNAs and stimulating decay and/or suppressing translation (Miller and Olivas, 2011a). Puf proteins are also involved in several aspects of neural function, such as neuronal excitability (Schweers *et al.*,

2002), dendrite morphogenesis in peripheral neurons (Ye *et al.*, 2004), and synaptic growth and plasticity at the neuromuscular junction (Menon *et al.*, 2004; Marrero *et al.*, 2011). Puf expression increases during long-term memory formation, and its disruption results in defective memory (Dubnau *et al.*, 2003).

The primary role of Puf proteins is to negatively regulate target mRNAs, although there are some cases in which Puf proteins positively regulate target mRNAs (Pique *et al.*, 2008; Archer *et al.*, 2009; Suh *et al.*, 2009). Sequences located in the 3' UTR of target mRNAs allow for dynamic assembly of ribonucleoprotein (mRNP) complexes consisting of Puf proteins, functional protein partners, and in some cases micro-RNAs. mRNP complex formation leads to functional changes in the mRNA such as decay or translational repression. The presence or absence of specific factors in mRNP complexes can determine the fate of an mRNA. Such factors include deadenylation and decapping proteins that influence the turnover of an mRNA. Also, translational repressors are assembled into mRNPs and can influence translation rate and efficiency. Concomitant with their function, Puf proteins are typically localized in the cytoplasm (Macdonald, 1992; Gallegos *et al.*, 1998; Crittenden *et al.*, 2002; Gerber *et al.*, 2004). In some cases, it is thought that Puf proteins localize to stress granules which are sites of mRNA turnover and/or mRNA storage during translational repression (Vessey *et al.*, 2006; Morris *et al.*, 2008; Balagopal and Parker, 2009). Other data suggests that Puf proteins can localize and repress on polysomes (Hu *et al.*, 2009). The specific localization of mRNA decay/translational repression is still largely debated; however, our research

suggests that Puf proteins in yeast are diffuse throughout the cytoplasm (Miller *et al.*, 2014) (Russo and Olivas, submitted).

Puf Protein Structure and Function

The Puf family is characterized by a conserved RNA-binding domain consisting of eight imperfect repeats of a 36 amino acid sequence, plus short flanking regions. This domain, also known as the Pumilio homology domain (Pum-HD) or the Puf repeat domain (PufRD), is located near the protein's C-terminus and is responsible for binding the 3'UTR of specific mRNAs (Goldstrohm *et al.*, 2006; Groban *et al.*, 2006; Padmanabhan and Richter, 2006; Deng *et al.*, 2008; Saint-Georges *et al.*, 2008; Ulbricht and Olivas, 2008; Kedde *et al.*, 2010; Ota *et al.*, 2011). For many Puf proteins, the PufRD alone is sufficient to regulate mRNA decay and repress translation (Houshmandi and Olivas, 2005; Padmanabhan and Richter, 2006; Kedde *et al.*, 2010). Regions outside the PufRD are not well conserved, although some Pufs contain glutamine-rich motifs, such as yeast Puf3p, which may promote aggregation (Miller and Olivas, 2011a). Recently, it has been shown that unique domains located in the N-terminus of *Drosophila* and human Pumilio proteins possess strong repressive capacity, suggesting a recently evolved regulatory function for domains outside of the repeat domain (Weidmann and Goldstrohm, 2012).

Target mRNAs contain a conserved Puf recognition element (PRE) consisting of a UGU sequence followed by an AU-rich downstream region (Souza *et al.*, 1999; Nakahata *et al.*, 2001; Tadauchi *et al.*, 2001; Wang *et al.*, 2001; White *et al.*, 2001; Wang *et al.*, 2002; Gerber *et al.*, 2004; Jackson *et al.*, 2004; Gerber *et al.*, 2006; Galgano *et al.*, 2008)

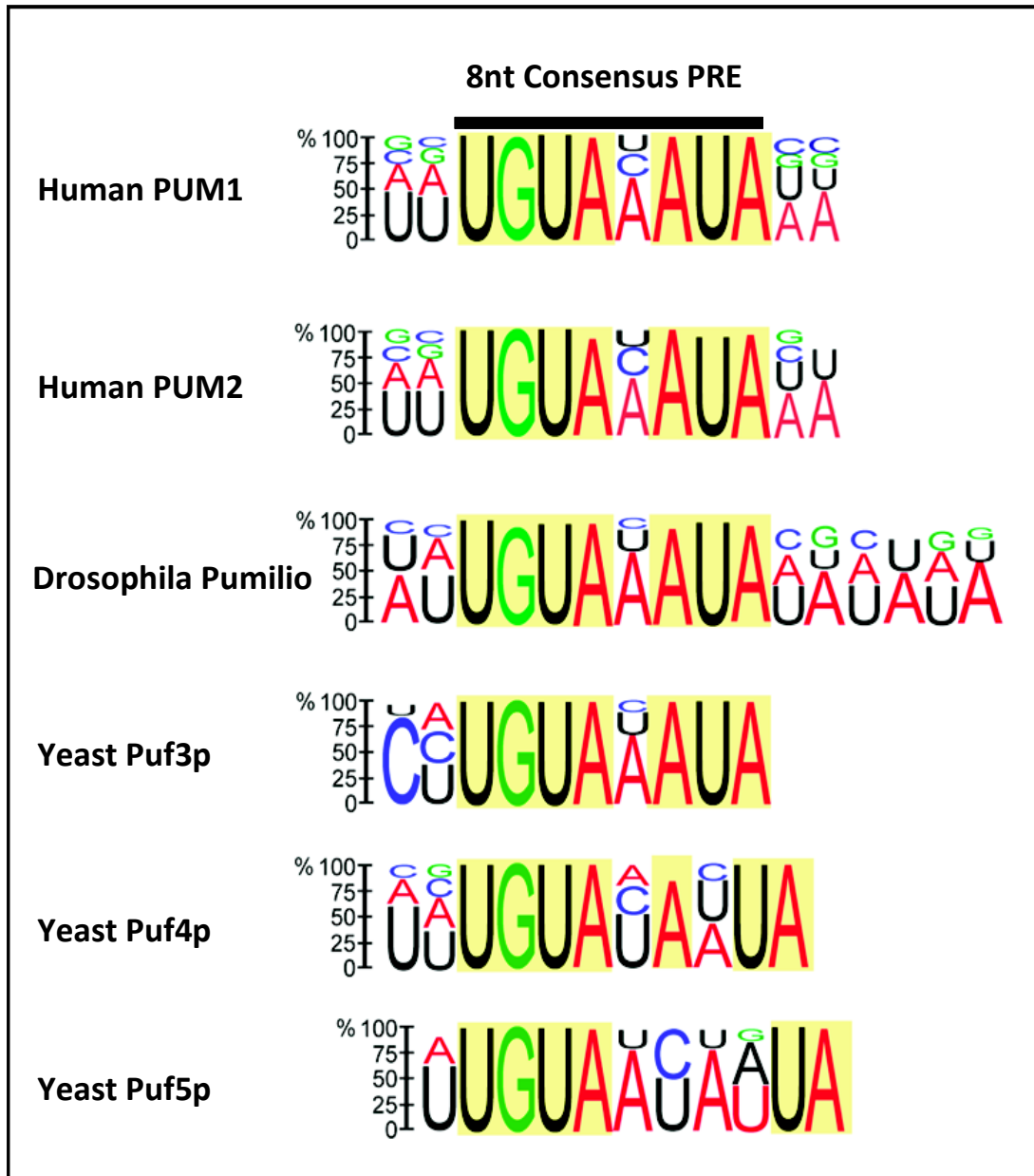


Figure 1.1. Puf protein binding element consensus sequences. Reprint from Miller and Olivas, 2011. Consensus sequence weight matrices were developed based on sequences in the 3' UTRs of mRNAs associated with human PUM1 and PUM2, *Drosophila Pumilio*, and *S. cerevisiae* Puf3p, Puf4p and Puf5p. The height of the nucleotide represents the probability that it will occur at that position. Positions that are conserved across organisms and proteins are highlighted in yellow (Gerber *et al.*, 2004; Gerber *et al.*, 2006; Galgano *et al.*, 2008)

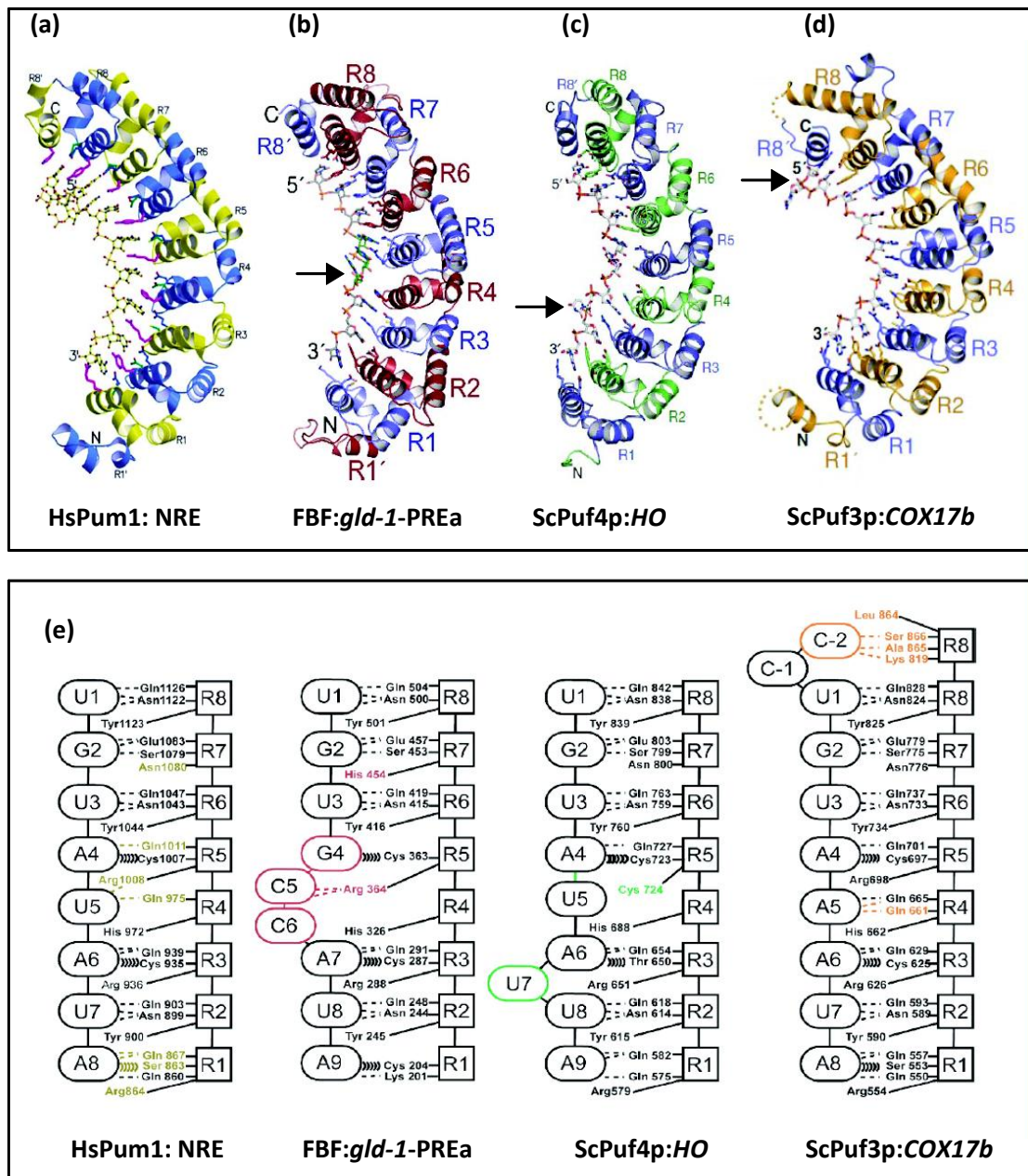


Figure 1.2. Puf protein binding schematics. Reprint from Miller and Olivas, 2011. Puf proteins bind recognition elements in either a one base to one repeat modular-manner or by inclusion of spacer/flipped bases. (a) Co-crystal structure of the canonical human PUM1-RD bound to a Nanos Response Element (NRE) from *Drosophila hunchback* mRNA (Wang *et al.*, 2002). (b) Co-crystal structure of *C.elegans* FBF-RD bound to a Puf Recognition Element (PRE) in *gld-1*. Arrow denotes flipped RNA base that does not interact with FBF amino acids (Wang *et al.*, 2009). (c) Co-crystal structure of yeast Puf4RDp bound to the *HO* PRE. Arrow denotes flipped RNA base that does not interact with Puf4p amino acids (Miller *et al.*, 2008). (d) Co-crystal structure of yeast Puf3RDp bound to a PRE in *COX17*. Arrow denotes novel interaction between an upstream cytosine base and amino acids of Puf repeat 8' (Zhu *et al.*, 2009). (e) Binding interactions between RNA bases and amino acids of PufRDs. Conserved interactions are represented in black. Amino acid–RNA base interactions and spacer/flipped bases that are unique to each Puf protein are indicated by color: *C.elegans* FBF (red), human PUM1 (gold), yeast Puf4p (green), and yeast Puf3p (orange) (Wang *et al.*, 2009).

(Figure 1). The PRE is typically between 8-10 nucleotides in length. Crystal structure analysis of several Puf-mRNA complexes reveals a primarily modular binding method, such that conserved amino acids within each repeat contact and stack with successive bases along the RNA in an anti-parallel manner, where bases 1-8 contact repeats 8-1 (Wang *et al.*, 2001; Wang *et al.*, 2002; Galgano *et al.*, 2008) (Figure 2). Puf proteins show strong binding specificity for targets; however, flexibility can involve RNA bases flipping out from the protein binding surface, allowing an increase in target mRNAs and possibly altering mRNP complex structure (Valley *et al.*, 2012). By flipping bases the local mRNA structure is changed, creating unique surface features that provide specificity. An interesting hypothesis is that the flipped bases may be sights of interaction for other regulatory proteins, providing another layer of regulation. An example of this can be seen with human Pum1, which can accommodate a 9-base binding site or an 8-base site (Miller and Olivas, 2011a). This feature greatly increases the number of potential target mRNAs and possibly offers an increase in protein binding partners. It is likely that Puf proteins are not as limited in their binding sites, aside from the core UGU. More research is needed to determine the binding capacity Pufs have for natural targets. Another feature that allows for specificity can be observed with yeast Puf3p. A cytosine at the -2 position from the UGU element is required for Puf3p binding *in vitro* and Puf3p decay regulation *in vivo* (Zhu *et al.*, 2009) (Figure 2e). Recent discoveries, including results presented herein, add to the promiscuity Puf proteins possess for RNA binding. Many of the mRNAs that associate with Puf proteins contain a similar binding motif

conserved across species; however, the targets themselves are functionally divergent between species.

Biological Roles of Puf Proteins and Their mRNA Targets

Puf proteins regulate diverse processes across eukaryotes. Although the recognition of mRNA targets is well conserved throughout eukaryotes, the function of the mRNAs is remarkably different. Considering this, it is hypothesized that the general role of Puf proteins is to support stem-cell maintenance and self-renewal (Wickens *et al.*, 2002; Miller and Olivas, 2011a). Included herein are multiple examples of the biological roles of Puf proteins and their mRNA targets in multiple organisms.

Through analysis of Puf mutant phenotypes across eukaryotes, many biological roles for Puf proteins have been elucidated and mRNA targets have been discovered. In *Drosophila*, Pumilio has been shown to regulate the mRNA *paralytic*, which controls neuron excitability and sodium current regulation. Pumilio mutant flies show hyper-excitability in motor neurons, which is attributed to an increase in *paralytic* mRNA; however, overexpression of Pumilio relieves the phenotype through repression of *paralytic* (Schweers *et al.*, 2002; Mee *et al.*, 2004; Muraro *et al.*, 2008). Also, through regulation of *eIF-4E* and *GluRIIA* mRNA, Pumilio regulates presynaptic growth and postsynaptic glutamate receptor subunit composition. An increase in the number of eIF-4E aggregates and increased expression of GluRIIA glutamate receptor can be seen in Pumilio mutants, suggesting Pumilio acts to repress these targets. Using gel mobility shift assays, the authors were able to validate Pumilio binding to these target RNAs, further supporting Pumilio's regulatory role (Menon *et al.*, 2004). Pumilio also regulates

posterior and anterior patterning through regulation of *hunchback* and *bicoid* mRNAs, respectively.

In *C. elegans*, FBF-1 regulates the translation of the mRNA *egl-4* determining plasticity in olfactory sensory neurons (Kaye *et al.*, 2009). FBF-1 and FBF-2 repress *fem-3* mRNA, which is required to control the spermatogenesis to oogenesis switch (Zhang *et al.*, 1997). Also, FBF-1 and FBF-2 regulate entry into meiosis by promoting *gld-1* expression (Crittenden *et al.*, 2002; Ariz *et al.*, 2009). PUF-5, PUF-6 and PUF-7 regulate egg shell formation/cytokinesis through multiple mechanisms, although the mRNA target(s) is/are not known (Lublin and Evans, 2007). PUF-8 regulates spermatogenesis, meiosis, and germline stem cell maintenance through regulation of *fog-2* expression, which acts upstream in germline sex determination (Subramaniam and Seydoux, 2003; Ariz *et al.*, 2009; Nadarajan *et al.*, 2009). PUF-9 controls hypodermal stem cell differentiation through regulation of *hbl-1* mRNA, a *let-7* miRNA target gene (Nolde *et al.*, 2007).

In *S. cerevisiae*, mutant phenotype analysis revealed Puf5p controls repression of filamentous-form cell differentiation (Prinz *et al.*, 2007). Puf5p also controls cell wall integrity, chronological and replicative life span through regulation of *LRG1* mRNA (Stewart *et al.*, 2007). Also, Puf5p controls peroxisome protein localization by regulating *PEX14* mRNA (Zipor *et al.*, 2009). *puf5* mutants show detergent sensitivity and susceptibility to the cell wall stain calcofluor white, suggesting defects in cell wall integrity (Kennedy *et al.*, 1995; Kennedy *et al.*, 1997; Kaeberlein and Guarente, 2002; Stewart *et al.*, 2007; Zipor *et al.*, 2009). *puf5* mutants also show increased stress

tolerance and an increased ability to go through mitosis multiple times before cell death (Kennedy *et al.*, 1995; Kennedy *et al.*, 1997). Puf5p and Puf4p regulate the mating-type switch by repressing expression of *HO* mRNA. Extensive gel shift study revealed two binding sites in the *HO* 3' UTR that are used by Puf4p and Puf5p (Hook *et al.*, 2007). Also, Puf1p and Puf5p repress *TIF1* mRNA, which encodes the translation initiation factor eIF4A, in a combinatorial manner (Ulbricht and Olivas, 2008). Puf1p, Puf4p and Puf5p regulate *HXK1* mRNA stability through multiple binding sites in the 3' UTR to control the hexokinase Hxk1 protein activity (Ulbricht and Olivas, 2008). Also, *YHB1* mRNA decay is regulated by Puf1p, Puf4p and Puf5p through one binding site to control Yhb1 protein levels for response to cellular nitrogen stress (this work). Puf3p regulates multiple mRNAs involved in mitochondrial biogenesis to respond to respiration requirements of the cell (Olivas and Parker, 2000; Eliyahu *et al.*, 2010; Gadir *et al.*, 2011; Miller *et al.*, 2013). Puf3 deletion strains show increased levels of many mRNAs encoding mitochondrial proteins, increased occurrence of aggregated mitochondria, and a decrease in mitochondria movement to the daughter bud tip (Olivas and Parker, 2000; Eliyahu *et al.*, 2010; Gadir *et al.*, 2011; Miller *et al.*, 2013). Overexpression of Puf3p in a non-fermentable carbon source also had growth defects, suggesting a disruption in respiratory function (Garcia-Rodriguez *et al.*, 2007).

In humans, Pum2 controls spermatogenesis through regulation of SDAD1 and CEP3 mRNAs (Urano *et al.*, 2005; Spik *et al.*, 2006). Also, Pum1 and Pum2 control cell proliferation by repressing the translation of the E2F3 transcription factor and enhancing the activity of E2F3 targeting miRNAs. PUM-1 also controls the cell cycle by

inducing local structure changes in the p27 mRNA, allowing for increased miR-221 and miR-222 accessibility to the 3' UTR for down-regulation. Pumilio knockdown led to a disrupted cell cycle phenotype (Kedde *et al.*, 2010).

It is abundantly clear that Puf proteins regulate a vast array of mRNA targets involved in many cellular functions among many different organisms. In any given organism there are multiple targets of Puf regulation. For example, yeast Puf3p alone stimulates the decay of at least 10 mRNAs with hundreds more predicted to be bona fide targets of Puf3p regulation (Olivas and Parker, 2000; Garcia-Rodriguez *et al.*, 2007; Miller *et al.*, 2013). Alternatively, a target mRNA can be regulated by multiple Puf proteins as observed with *HO*, *TIF1*, *HXK1* and *YHB1* mRNAs in yeast (Hook *et al.*, 2007; Ulbricht and Olivas, 2008) (this work). Only one Puf protein can be bound at a binding site at a given time thus this combinatorial control allows for redundancy ensuring the availability of Pufs to regulate the target. Furthermore, many targets of Puf regulation contain multiple binding sites that are separated enough to allow each site to be occupied by a Puf, suggesting the importance of combinatorial regulation. Multiple Puf regulation of one target mRNA may also allow control under conditions that inactivate particular Pufs or permit use of different mechanisms to regulate the mRNA.

Global analysis of mRNAs that co-purify with Puf proteins suggest specific classes of functionally related mRNAs are bound by different Pufs (Gerber *et al.*, 2004; Gerber *et al.*, 2006; Galgano *et al.*, 2008). This supports the RNA regulon theory that suggests that functionally related RNAs are co-regulated by interactions with specific trans-factors to allow for post-transcriptional regulation (Keene, 2007; Kanitz and Gerber,

2009; Morris *et al.*, 2009). In a given organism, Puf bound targets are often involved in functions in a specific subcellular compartment, organelle or regulatory pathway. In *S. cerevisiae*, affinity-tagged purification of Puf1p-Puf5p revealed interactions with mRNAs that function in distinct subcellular compartments or organelles. For example, Puf1p and Puf2p bind mRNAs coding for membrane-associated proteins, Puf3p binds mRNAs that encode mitochondrial proteins, and Puf4p and Puf5p bind mRNAs that function in the nucleus (Gerber *et al.*, 2004). Another example can be found with *Trypanosoma brucei*, where purified PUF9 was enriched for mRNAs having a role in DNA replication (Archer *et al.*, 2009). Finally, *C. elegans* FBF associates with transcripts involved in meiosis, the Ras/MAPK pathway, apoptosis and stem cell maintenance (Kershner and Kimble, 2010).

Many of the mRNAs that associate with human PUM1 and PUM2, *Drosophila* Pumilio and yeast Puf3p have a very similar binding site; however, these bound mRNAs are often not conserved between organisms when homologs exist (Gerber *et al.*, 2006; Galgano *et al.*, 2008; Morris *et al.*, 2008). Although target mRNAs are not well conserved across species, Puf-mediated control of some biological processes is conserved across species, providing evidence for evolutionary conservation of Puf function.

Mechanisms of mRNA Repression

There are numerous ways in which Puf proteins could repress protein expression of target mRNAs. Puf proteins may simply be a scaffold for other proteins that are active in repression. Alternatively, Puf proteins themselves could have repressive

function by blocking translation or recruiting decay machinery. Puf proteins could alter local structure to allow access to previous inaccessible locations for protein binding or miRNA binding. Research suggests that all of these repression mechanisms can and do occur. Specifically, once bound to a target mRNA, Puf proteins elicit repression either through protein interactions that limit cap-binding events to inhibit translation initiation, or interactions with mRNA decay machinery to stimulate deadenylation and decapping steps of decay (Goldstrohm *et al.*, 2006; Goldstrohm *et al.*, 2007; Miller and Olivas, 2011b).

Several examples of different mechanisms of repression exist in multiple organisms. A conserved Puf mechanism is stimulation of mRNA decay by recruitment of the CCR4-POP2-NOT deadenylase complex. Puf proteins in yeast, *Drosophila*, *C. elegans* and humans recruit and directly bind Pop2, which acts as a bridge molecule between Pufs, CCR4 and the NOT complex (Goldstrohm *et al.*, 2006; Goldstrohm *et al.*, 2007; Hook *et al.*, 2007; Lee *et al.*, 2010) (this work) (Figure 1.3(a)). The presence of Ccr4p, the catalytic subunit of the deadenylation complex, results in deadenylation of the target mRNA (Goldstrohm *et al.*, 2006; Goldstrohm *et al.*, 2007; Hook *et al.*, 2007). The Puf-Pop2 interaction is conserved in humans, illustrating the importance of this mechanism for Puf mediated decay (Van Etten *et al.*, 2012). The resulting deadenylated target mRNA subsequently has its cap removed by decapping factors and is then rapidly decayed in the 5' to 3' direction by exonucleases (Muhlrads *et al.*, 1994) (Figure 1.3 (a)). Recently, Pufs have been shown to use the repeat domain to antagonize poly A binding protein (PABP) to accelerate deadenylation (Weidmann *et al.*, 2014).

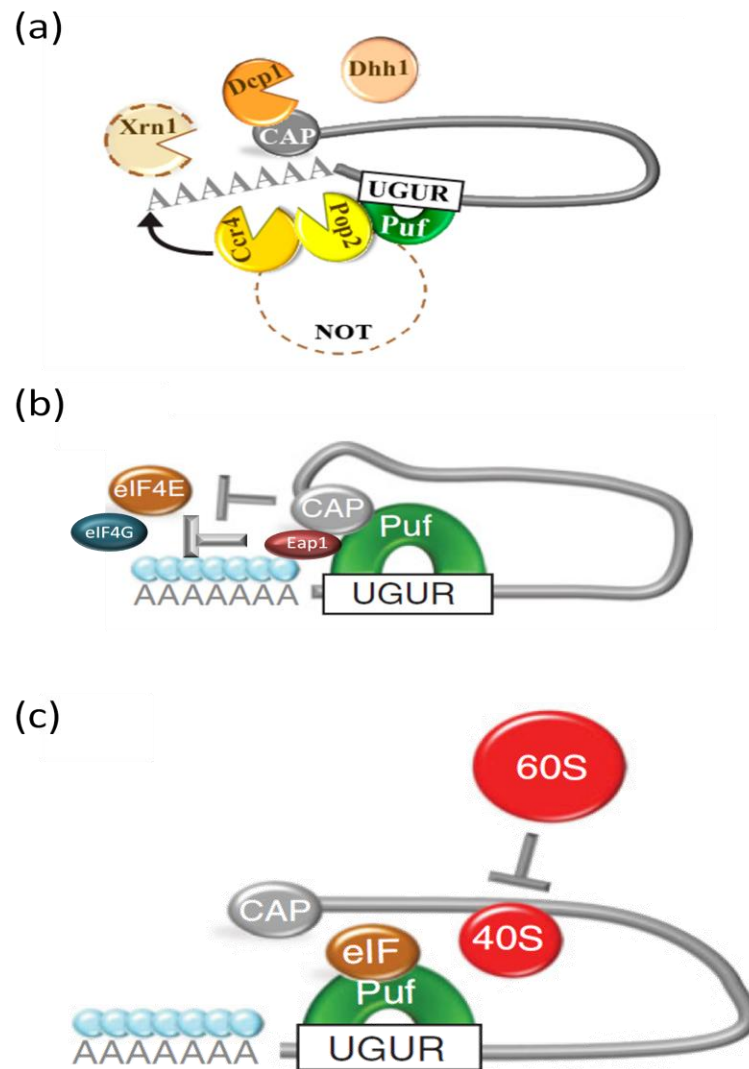


Figure 1.3. Modes of Puf-mediated regulation. Reprinted for Miller and Olivas, 2011 with modifications. Translational repression and decay mechanisms through direct interactions with Puf proteins. (a) Pufs directly recruit the Pop2p subunit of the Ccr4p-Pop2p-Notp deadenylase complex, which in turn binds Ccr4p and presumably the rest of the Not complex. The Puf protein also recruits Dcp1p, which cleaves the 5' cap, and Dhh1, a regulator of mRNA degradation (Kennedy *et al.*, 1995; Kennedy *et al.*, 1997; Goldstrohm *et al.*, 2006; Goldstrohm *et al.*, 2007; Hook *et al.*, 2007; Stewart *et al.*, 2007). After deadenylation and decapping of the transcript, the 5'→3' exonuclease Xrn1p rapidly degrades the mRNA (Muhlrad *et al.*, 1994). (b) Pufs inhibit translation by competing with eIF4E for interaction with the mRNA cap or by competing with initiation factor eIF4G to disrupt binding to eIF4E (Cao *et al.*, 2010; Blewett and Goldstrohm, 2012). (c) Pufs can also repress translation of an mRNA by either interacting with specific translation factors such as eIF5B or by competing with them for interactions with other translation initiation factors. Pufs can also prevent the formation of the 80s ribosome complex (Deng *et al.*, 2008).

Puf proteins can also repress protein expression by disrupting translation. Removal of the poly-A tail can inhibit translation itself; however, Puf proteins accomplish translational inhibition in more direct ways as well. Pufs can disrupt mRNA interactions with the translational initiation machinery. For example, yeast Puf6p represses ASH1 mRNA translation by interacting with Fun12p/eIF5B or by competing for interactions with different initiation factors (Deng *et al.*, 2008). In vitro translation assays indicate Puf6p also prevents the formation of the 80s ribosome complex during translation (Deng *et al.*, 2008) (Figure 1.3 (c).) Yeast Puf5p requires interactions with translational repressor Eap1p to efficiently decap and repress target mRNAs in addition to competing with initiation factor eIF4E for cap binding. (Blewett and Goldstrohm, 2012) (Figure 1.3 (b)). Also, human Pumilio can repress independent of deadenylation, as seen on a target lacking a Poly-A tail, likely through translational repression (Van Etten *et al.*, 2012).

Higher eukaryotes use many auxiliary proteins, such as BRAT, Nanos, CPEB, DAZ and DAZL, to function with Puf proteins to accomplish mRNA repression. The requirement for these protein partners depend on the organism and the mRNA target because homologs are not conserved across eukaryotes; however, Nanos homologs have been identified in *Drosophila*, humans, *C. elegans*, and *Xenopus*, and are required for the repression of many mRNA targets (Sonoda and Wharton, 2001; Cho *et al.*, 2006; Kadyrova *et al.*, 2007). Nanos can interact specifically (Kadyrova *et al.*, 2007) or non-specifically (Curtis *et al.*, 1997; Kraemer *et al.*, 1999; Sonoda and Wharton, 1999; Nakahata *et al.*, 2001; Jaruzelska *et al.*, 2003) with transcripts and directly interact

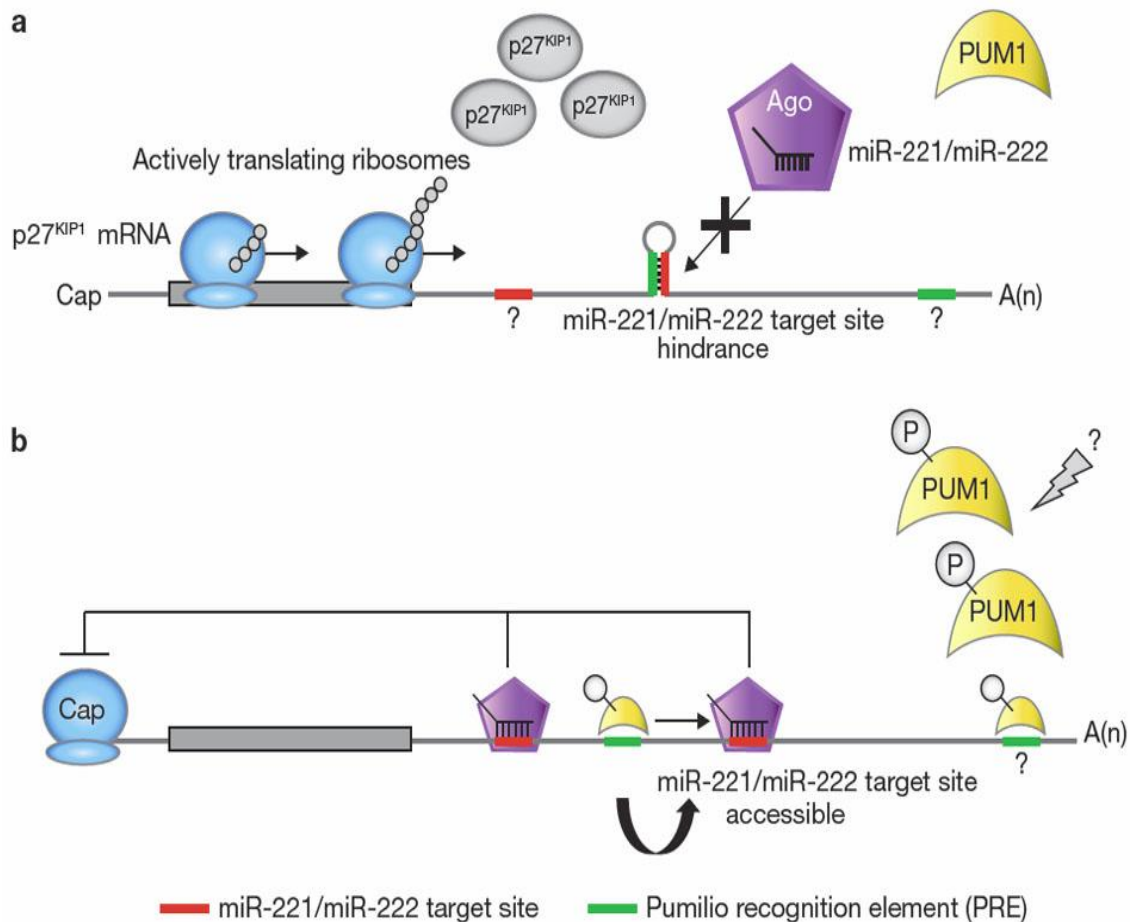


Figure 1.4. Mode of Pumilio activity in humans. Reprint from Triboulet and Gregory, 2010. **(a)** In quiescent fibroblasts, *p27* mRNA is actively translated to yield high levels of p27 protein. One of the two target sites for miR-221/miR-222 in the *p27* 3' UTR is embedded in a stable stem-loop structure together with one of the two conserved Pumilio recognition elements (PREs), thus preventing *p27* silencing by miR-221/miR-222. **(b)** When cells re-enter the cell cycle on growth-factor stimulation, levels of Pumilio protein PUM1 increase and phosphorylation of the Ser 714 enhances its RNA-binding activity. PUM1 binds to the proximal PRE to induce a local change in the RNA that enables miR-221/miR-222 binding to its target site and repression of *p27* translation. Ago; Argonaute. (Galgano *et al.*, 2008; Triboulet and Gregory, 2010)

with PufRD to stabilize repression complexes. In *Drosophila*, translational repression of some mRNAs, such as *hunchback*, requires the formation of complexes containing Brat, Nanos, Pumilio, and the target mRNA (Wreden *et al.*, 1997; Sonoda and Wharton, 1999, 2001); however, other complexes exclude Brat to perform repression (Sonoda and Wharton, 2001; Kadyrova *et al.*, 2007). Human PUM2 interacts with several Puf protein partners, such as the Deleted in Azoospermia (DAZ), Deleted in Azoospermia-Like (DAZL), and the BOULE (BOL) RNA-binding protein family (Urano *et al.*; Fox *et al.*) The ability of Puf proteins to bind different partners such as Nanos, DAZ, DAZL, and BOL allows for mRNP structure changes that can use different mechanisms for mRNA repression, increasing the complexity of regulation by Puf proteins.

Recently, Puf proteins have also been found to act cooperatively with the miRNA regulatory system. Global studies to identify mRNAs bound by human Pum1 and Pum2 determined that 3' UTRs containing Puf binding sites are enriched in miRNA binding sites (Galgano *et al.*, 2008). In humans, miRNA sites located near Puf binding sites are often located in highly structured 3' UTR regions that are poorly accessible, and the distance between the miRNA and Puf sites is typically less than 50 nucleotides (Leibovich *et al.*, 2010). For example, Puf binding sites are often associated with miR-410 target sites located in highly structured regions (Leibovich *et al.*, 2010). Experimentally, binding of human Pum1 to the 3' UTR of the p27 tumor suppressor mRNA was found to alter the local RNA structure and permit binding of miR-221 and miR-222 for RNA repression (Figure 1.4) (Kedde *et al.*, 2010). Binding of human Puf proteins to the 3' UTR of the E2F3 oncogene also enhanced miRNA-mediated repression

of this mRNA, and several types of cancer circumvent miRNA regulation by shortening the 3' UTR, thereby eliminating the Puf binding site (Miles *et al.*, 2012). In addition to making miRNA sites more accessible, Pufs may also work coordinately with miRNAs through interactions with the miRNA complex protein Argonaute, and these interactions have been shown to inhibit translation elongation (Friend *et al.*, 2012).

Regulation of Puf Protein Activity

Puf proteins regulate functionally related mRNAs in a coordinated manner to achieve protein expression goals required by the cell. Although Puf proteins repress mRNAs, it is not likely that they repress target mRNAs at all times. It is more likely that Puf protein activity is responsive to environmental stimuli or cellular signaling during precise times when a rapid expression control mechanism is needed. Such control over Puf protein activity would allow for use of already existing mRNA pools to respond to stimuli. A model for this hypothesis is to alter Puf protein activity and/or expression levels to repress or derepress target mRNAs as needed. Indeed, eukaryotes have developed multiple ways to regulate Puf activity such as modulating expression levels, disrupting the ability to interact with target mRNAs, and disrupting interactions with co-factors. Puf proteins are controlled at all levels of gene regulation including transcription, post-transcription, translation and post-translation, suggesting activity is tightly controlled.

In *C. elegans*, it is thought that an autoregulatory mechanism exists for post-transcriptionally regulating *fbf-1* and *fbf-2* mRNA levels through binding of FBF proteins to their 3' UTRs. Also, in humans both Pum1 and Pum2 mRNA bind purified Pum1

protein, indicating that this autoregulation may be conserved (Morris *et al.*, 2008). Puf expression is also regulated post-transcriptionally through the miRNA pathway. In rat, PUM2 has been shown to be negatively regulated by miR-134, and interestingly the 3' UTRs of PUM2 homologs in mouse and human also contain miR-134 binding sites, again suggesting conservation of this mechanism (Fiore *et al.*, 2009). Recently, miR-340 has been implicated in lung cancer progression through regulation of Pum1 and Pum2 in humans, further supporting post-transcriptional control of Puf protein expression and activity (Fernandez *et al.*, 2014).

Puf protein activity may also be altered at the post-translational level. In budding yeast, phosphorylation of Puf6p by protein kinase CK2 inhibits translational repression by Puf6p (Deng *et al.*, 2008). In humans, Pum1 RNA-binding activity to the p27 3' UTR is dependent on phosphorylation of Pum1 in response to growth factor stimulation (Figure 1.4) (Kedde *et al.*, 2010). Work in the Olivas lab has shown that Puf protein activity in yeast is active during cellular fermentation and inactive during cellular respiration. This activation/inactivation occurs rapidly, within two minutes, during metabolic changes from fermentation to respiration or vice versa suggesting that a post-translational modification may be responsible for altered activity. The binding of yeast Puf1p, Puf3p, and Puf4p to their target mRNAs is not disrupted in fermentation versus respiration conditions, while Puf5p is less able to bind target mRNAs in respiration conditions (Miller *et al.*, 2013) (This Work).

A final way Puf protein activity is regulated is through interactions with binding partners. In *Drosophila*, Bam and Bgcn form a complex with Pumilio where Bam directly

interacts with the N-terminal region of Pumilio, and this binding abolishes repressive activities of the Pumilio repeat domain (Kim *et al.*, 2010). In this work, yeast Puf3RD is shown to have limited binding capacity for POP2p in inactivating conditions (respiration). Together, this research provides mechanistic details into how Puf protein activity is controlled.

Contribution to the Field of Puf Research

The research presented herein provides new insight into Puf protein regulation. In one project, I identify *YHB1* mRNA as a bona fide target of Puf1p, Puf4p and Puf5p regulation in yeast. This mRNA utilizes a single, highly flexible PRE to bind three different Puf proteins to accomplish regulation. Each individual Puf protein is able to regulate *YHB1* mRNA in the absence of the other Puf proteins. Puf1p, Puf4p and Puf5p activity is tightly regulated for *YHB1* mRNA decay, as alterations in the growth carbon source affects Puf activity. Specifically, growth in dextrose allows for repressive Puf activity; however, growth in galactose inhibits Puf activity completely. This result is also seen with a second target of Puf1p, Puf4p and Puf5p regulation, *HXK1* mRNA. Overexpression of Puf4p or Puf5p in dextrose conditions increases the repression capacity beyond that of wild-type, suggesting the amount of Puf proteins is limited in the cell. Overexpression of Pufs in galactose can rescue inactivation of Puf proteins suggesting that the inactivation signal is limiting as well. Furthermore, Puf4p overexpression reduces the steady-state levels of *YHB1* mRNA during nitric oxide exposure, resulting in a growth defect and further illustrating the importance of “fine-tuned” protein expression control in response to environmental stress. The differential

activity of Puf1p, Puf4p and Puf5p in different carbon sources is not a result of altered Puf protein expression levels or changes in localization. Rather, Puf5p has a reduced ability to bind target mRNAs, although this is not the case with Puf1p or Puf4p. I also identified a Puf5p mutant that has increased repressive capacity compared to wild-type, potentially offering mechanistic insight into Puf protein regulation.

In a second project, I will present mechanistic information on Puf3p condition-specific activity in yeast. Puf3p was the first Puf identified in the lab as being inactivated in respiratory conditions such as galactose and ethanol. I determined that Puf3p's altered activity in galactose is not due to altered localization or differential mRNA binding. In fact, binding to Puf3p target mRNA *COX17* was enhanced in galactose. Instead, a potential mechanism for altered Puf3p activity is to change the local mRNP complex. Indeed, during growth in galactose conditions Puf3RD was limited in its ability to bind Pop2p. Further evaluation of Puf3p decay complex formation in a POP2 Δ strain revealed that cofactors such as Ccr4p, Dhh1p and Dcp1p are no longer able to bind in the absence of Pop2p, further supporting Pop2p as a crucial cofactor for Puf3p activity. His-repression assays further support the notion that Puf3p repression is disabled in the absence of Pop2p. Also, I identified the mechanism by which a Puf3RD mutant is unable to accomplish mRNA decay. This mutant has a loop structure disrupted between repeats 7 and 8 such that it can still bind target mRNAs but cannot accomplish mRNA decay. Interactions with full-length Pop2p are disrupted with this Puf3RD loop mutant, instead binding to a truncated form of Pop2p occurs. The nature of this smaller Pop2p is yet to be elucidated; however, it is clear that the mutant Puf3RD cannot bind the functional,

full-length Pop2p. Mass-spectra comparison between the two protein forms will provide critical information on how repression is affected by these two Pop2p species. I am performing research into Pop2p mutants that block or mimic a known phosphorylation state to determine if this post-translational modification is responsible for altered binding to Puf3RD. Yak1p phosphorylates Pop2p in the absence of dextrose, (Moriya *et al.*, 2001) and we show that Puf3p activity is retained in a *YAK1Δ* strain in the presence of galactose. This data suggests that galactose provides an inactivating signal to Puf3p through Pop2p phosphorylation, wherein phosphorylation of Pop2p may inhibit the ability of Puf3RD to interact with Pop2p. Furthermore, this data provides a potential mechanism for carbon-source dependent regulation of mRNA decay by Puf3p.

Finally, I have used my knowledge of Puf proteins in yeast to move into research on human Pumilio proteins. The Olivas lab has previously focused on using yeast as a model organism for studying Puf proteins. I have transitioned the lab into using human cell culture for studies of Pumilio proteins.

My third project presented herein discusses the discovery of novel targets of Pum1 and Pum2 regulation in humans. I have discovered that several genes implicated in Parkinson's disease are targets of Pumilio regulation. Specifically, SAT1, SNCA, and LRRK2 are identified as targets of Pumilio regulation. In doing so, I have established protein knockdown protocols for the lab as well as standard cell culture methods for two different cell lines. Also new to the lab are methods for transfection of reporter plasmids for luciferase-based assays. Establishing these techniques indicates that our

lab is capable of performing these types of experiments and is critical for future funding opportunities.

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**CHAPTER 2: CARBON SOURCE-DEPENDENT INTERACTIONS OF
PUF3P WITH POP2P DETERMINE TARGET mRNA REPRESSION**

INTRODUCTION

Precise control of protein production is critical to proper cellular function and is needed to respond to a variety of environmental stimuli. Cells control protein production in a variety of ways including transcriptional regulation, post-transcriptional regulation, translational regulation and post-translational regulation. One such post-transcriptional mechanism is regulation of mRNA decay. Precise regulation of mRNA decay offers a mechanism to respond rapidly to environmental stresses by altering the use of already available mRNA (Shim and Karin, 2002). Regulation of mRNA decay is often accomplished by RNA binding proteins that recognize cis-elements located in the 3' untranslated region (3' UTR) of mRNAs.

The Puf family of RNA binding proteins regulate target mRNAs by binding cis-acting regulatory elements contained within the 3' UTR. Typically, Puf proteins bind conserved UGU elements, leading to recruitment of factors that disrupt translation initiation and/or promote mRNA degradation (Miller and Olivas, 2011). Puf proteins regulate assorted cellular processes including memory formation, embryonic development, organelle biogenesis and stress response (Barker *et al.*, 1992; Wickens *et al.*, 2002; Spassov and Jurecic, 2003; Wharton and Aggarwal, 2006; Miller *et al.*, 2014). Although the binding sequence is conserved in eukaryotes, target mRNAs can vary between organisms and particular mRNAs may not be conserved between organisms even when orthologs exist (Gerber *et al.*, 2006; Galgano *et al.*, 2008; Morris *et al.*, 2008). However, targets of specific Puf proteins are often mRNAs that are functionally related

and therefore are functionally regulated (Foat *et al.*, 2005). This can be observed with *Saccharomyces cerevisiae* Puf3p, which binds target mRNAs that encode mitochondrial proteins (Gerber *et al.*, 2004; Miller *et al.*, 2014).

Although Puf proteins such as *S. cerevisiae* Puf3p act to stimulate decay of their target mRNAs, these mRNAs are not repressed at all times. Target mRNAs are likely repressed when the cell needs a limited amount of protein product; however, when an increase in protein product is required, target mRNAs are derepressed. Puf protein activity is altered to accommodate cellular protein requirements. For example, human Pum1 RNA-binding activity to the p27 3' UTR is dependent on phosphorylation of Pum1 in response to growth factor stimulation (Kedde *et al.*, 2010). In *C. elegans*, current models suggest an autoregulatory mechanism for post-transcriptionally regulating *fbf-1* and *fbf-2* mRNA levels by binding of the FBF proteins to their own 3' UTRs (Lamont *et al.*, 2004) and this mechanism may be conserved in humans (Morris *et al.*, 2008). In budding yeast, Puf6p N-terminal phosphorylation by CK2 causes translational derepression (Deng *et al.*, 2008). mRNAs that are targeted for decay by Puf1p, Puf3p, Puf4p and Puf5p in glucose conditions are stabilized in response to glucose deprivation (Miller *et al.*, 2014) (Russo and Olivas, submitted). Specifically, Puf3p regulates mitochondrial biogenesis and function by promoting rapid degradation of target mRNAs in fermentable conditions (glucose/dextrose-these are synonymous) where the need for mitochondria is low; however, in respiration conditions such as galactose where mitochondria production is needed, Puf3p decay activity is inhibited allowing for increased protein production for mitochondria biogenesis. Additionally, Puf1p, Puf4p

and Puf5p stimulated decay of *YHB1* mRNA in glucose conditions, but *YHB1* mRNA is stabilized in response to increased accumulation of toxic nitric oxide from respiration (Russo and Olivas, submitted). Altered mRNA binding is at least partially responsible for the reduction in Puf5p activity (Russo and Olivas, submitted); however, the mechanism for reduced activity in Puf1p, Puf3p and Puf4p has remained elusive. Our research indicates that reduced activity is not a result of altered protein expression, localization or RNA binding. In fact, Puf3p enhances its binding capacity in galactose conditions (Miller *et al.*, 2014), further supporting a dual role for Puf3p in localizing target mRNAs for localized protein synthesis (Eliyahu *et al.*, 2010; Gadir *et al.*, 2011). Thus, the observed inactivation of Pufs in yeast may be due to altered interactions with decay machinery or other inhibitory factors. Precedent for this mechanism comes from *C. elegans* and *Drosophila* where GLD3, Bam and Bgcn proteins bind Puf proteins to inhibit their repressive activity (Eckmann *et al.*, 2002; Kim *et al.*, 2010).

Puf proteins in yeast utilize the recruitment of mRNA decay machinery to facilitate degradation. Key members of the decay machinery include the deadenylation complex consisting of Pop2p, Ccr4p and the NOT complex. Also included is the decapping complex consisting of Dcp1p/Dcp2p and the enhancer Dhh1p. Puf proteins directly bind Pop2p, which likely acts as a bridge for binding other decay factors including Ccr4p, Dcp1, Dhh1 and the NOT complex (Goldstrohm *et al.*, 2006; Goldstrohm *et al.*, 2007; Hook *et al.*, 2007; Lee *et al.*, 2010) (this work). Recruitment of the catalytically active subunit of the deadenylation complex, Ccr4p, by Puf proteins results in deadenylation of the mRNA (Goldstrohm *et al.*, 2007; Lee *et al.*, 2010). The mRNA is

then decapped by Dcp1p/Dcp2p and subjected to 5' to 3' exonuclease degradation by Xrn1p.

In this work I have identified a condition-specific mechanism for control of Puf3p activity. We identified deadenylation as the key regulatory step that is inhibited in respiration conditions. Furthermore, Puf3p interactions with Pop2p are greatly reduced during respiration, supporting inhibition of deadenylation as a key regulatory step. We provide *in vivo* evidence of the requirement of Pop2p for bridging protein interactions with Ccr4p, Dhh1p and Dcp1p. Additionally, the kinase Yak1p has been shown to rapidly phosphorylate Pop2p in response to glucose deprivation, and this modification is removed rapidly (within 1 minute) upon glucose addition (Moriya *et al.*, 2001). Transcriptional shut-off assays in a *yak1Δ* strain revealed that Puf3p remains active for decay of *COX17* mRNA in galactose suggesting that phosphorylation of Pop2p disrupts binding with Puf3p and/or function for Puf3p mediated decay stimulation. Finally we identified a truncated form of Pop2p that interacts with Puf3RD more predominantly than the wild-type Pop2p in respiration conditions. Furthermore, the smaller Pop2p almost exclusively interacts with our Puf3RD loop 7a mutant in fermentation conditions. This Puf3RD loop 7a mutant is capable of binding target mRNAs; however, it is unable to facilitate decay (Houshmandi and Olivas, 2005). This exclusive binding of the mutant Puf3RD with the truncated form of Pop2p further supports the hypothesis that this interaction inhibits mRNA decay. Finally, mass spectrometry analysis of the two species of Pop2p suggests an N-terminal truncation of the full length Pop2p for formation of the smaller version of Pop2p. These results define a condition-specific mechanism by which

altered Puf3p interactions with Pop2p results in inactivation of Puf-mediated mRNA decay.

RESULTS AND DISCUSSION FROM PREVIOUS STUDY (MILLER ET AL, NAR 2014)

I was a second author on a paper from our lab entitled “Carbon source-dependent alteration of Puf3p activity mediates rapid changes in the stabilities of mRNAs involved in mitochondrial function”. Together with Anthony Fischer, I performed the analysis of Puf3p conditional localization as well as *in vivo* RNA-immunoprecipitation studies to evaluate conditional mRNA binding capacity. The following describes and discusses that work.

Condition-specific inactivation of Puf3p is not due to altered localization

To analyze Puf3p localization under different conditions, endogenously GFP-tagged Puf3p was visualized in cells grown in dextrose, galactose or ethanol conditions. Similar to the results seen by western analysis, Puf3p-GFP levels detected microscopically were increased >1.5-fold in galactose and ethanol as compared with dextrose (Figure 2.1). Despite differences in expression levels, Puf3p-GFP was diffusely localized across the cytoplasm with multiple small foci in all conditions, demonstrating that localization is not markedly altered by inactivating conditions (Figure 2.1). Previous work has shown a similar localization pattern in dextrose (Garcia-Rodriguez *et al.*, 2007). Specifically, Puf3p does not exclusively localize to mitochondria in galactose or ethanol, though some Puf3p does appear to localize to the perimeter of mitochondria as seen by the yellow rings surrounding the red mitochondrial foci in the merged images between

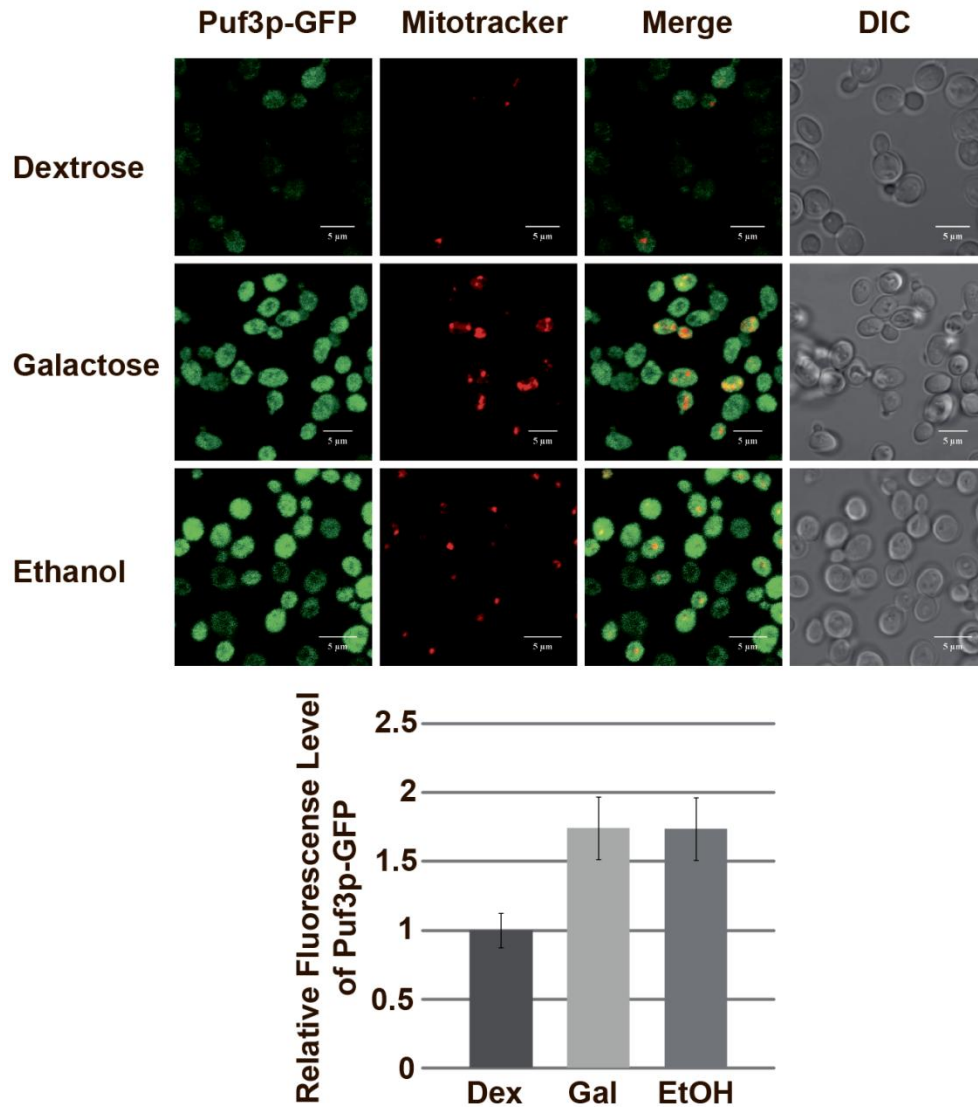


Figure 2.1. Ethanol and galactose do not decrease PUF3 expression levels or alter localization. Yeast expressing endogenously GFP-tagged Puf3p were grown in YEP media supplemented with 2% dextrose, galactose or ethanol. Each image represents 10 flattened Z plane slices through fixed cells using a confocal fluorescence microscope. Puf3p-GFP is shown in green, while mitochondria stained with Mitotracker Deep Red are shown in red. Merge indicates merger of the Puf3p-GFP and Mitotracker windows. Bright field images of each cell field are shown. The bar equals 5mm. Fluorescence levels of Puf3p-GFP, quantified across cell fields and normalized to levels in dextrose, are graphically represented.

Puf3p-GFP and Mitotracker red (Figure 2.1). This result supports previous studies indicating that Puf3p localizes to the cytosolic face of mitochondria (Garcia-Rodriguez *et al.*, 2007).

Condition-specific inactivation of Puf3p does not disrupt Puf3p-mRNA interactions

The condition-specific regulation of Puf3p could be altering its ability to associate with its mRNA targets or its ability to functionally stimulate decay of the targets. To address whether Puf3p-mRNA interactions are disrupted under inactivating conditions, qPCR was used to quantitate the amount of mRNA that copurified with endogenously TAP-tagged Puf3p from cells grown in dextrose or galactose. We tested *COX17* mRNA as a positive target of Puf3p regulation, as well as *CBS1* and *STE3* mRNAs as negative controls to ensure we specifically enriched for Puf3p binding targets after the IP process. As shown in Figure 2.2 A and B, *COX17* mRNA was enriched 200- to 800-fold following Puf3p IP from both galactose and dextrose conditions as compared with input mRNA levels, while there was no enrichment of either *CBS1* or *STE3* mRNA. Having demonstrated specificity of Puf3p binding, we next compared levels of *COX17* that copurified with Puf3p from dextrose and galactose conditions. *COX17* mRNA levels from the IP were >2-fold higher in galactose versus dextrose, indicating that Puf3p-mRNA interactions are not disrupted under conditions that inactivate Puf3p-mediated decay stimulation (Figure 2.2C). The higher level of *COX17* mRNA that copurified with Puf3p in galactose likely reflects the increased amount of Puf3p expressed in galactose conditions (Figure 2.2 D). These results support the hypothesis from previous studies that Puf3p binds and traffics its mRNA targets to the mitochondria (Saint-Georges *et al.*,

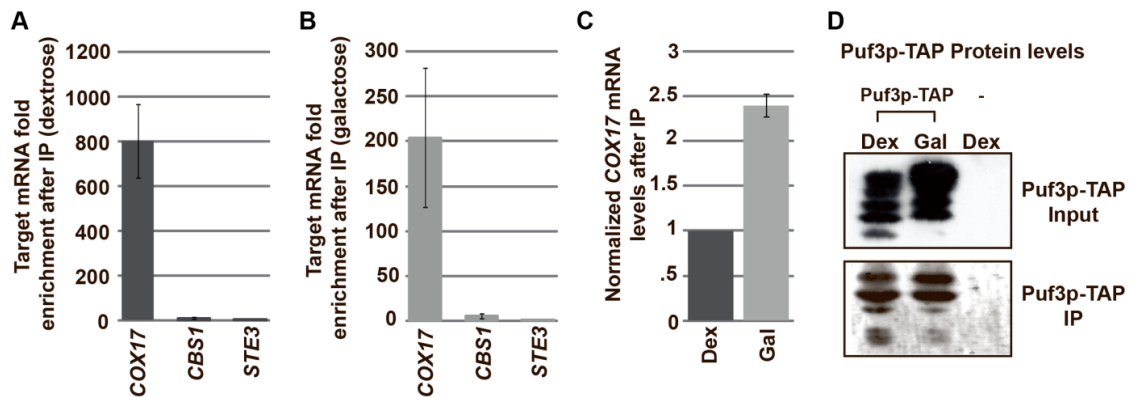


Figure 2.2. Puf3p binds its target mRNAs in both activating and inactivating conditions.

Endogenously TAP-tagged Puf3p was immunoprecipitated (IP) from yeast grown in YEP media supplemented with 2% dextrose or galactose using IgG-Sepharose, and RNAs associated with Puf3p were analyzed by qPCR. Fold mRNA enrichment after IP from dextrose (A) or galactose (B) conditions was calculated by comparing average Cq values of mRNAs isolated after IP versus average Cq values of the respective mRNAs isolated from total cell lysate before IP. (C) Average Cq values of mRNAs isolated after IP were compared between dextrose and galactose conditions, with levels normalized to that found in dextrose. (D) Puf3p-TAP protein levels from dextrose and galactose conditions in total cell lysates (Input) and after IP were assessed by western blot using anti-TAP antibodies. A strain expressing nontagged Puf3p (-lane) is also shown.

2008; Gadir *et al.*, 2011), and our results demonstrate that such binding occurs regardless of the conditions. In contrast, the inactivation of Puf3p is likely disrupting its ability to stimulate decay of the bound targets.

Discussion of possible mechanisms of Puf3p inactivation in respiration conditions

Our data showing rapid changes in Puf3p activity upon carbon source shift without carbon source-dependent changes in Puf3p expression suggest that changes in Puf3p decay activity are regulated posttranslationally. One possibility is that Puf3p is sequestered into aggregates or its localization is otherwise altered to render it inactive. However, our results indicate that localization is not significantly altered between conditions, being diffusely localized with multiple small foci throughout the cytoplasm (Figure 2.1). A second possibility is that phosphorylation modulates Puf3p activity, as Puf6p-mediated translational repression is regulated by CK2 phosphorylation (Deng *et al.*, 2008) . Mass spectrometry analysis of condition-dependent Puf3p phosphorylation has to date been inconclusive (Anthony Fischer, unpublished data). Activity may also be altered by changes in proteins that interact with Puf3p. In addition to its role in stimulating mRNA decay, Puf3p stimulates mitochondrial localization of nuclear-transcribed mRNAs containing Puf3p binding sites. In a *puf3Δ* strain grown in galactose (Saint-Georges *et al.*, 2008) or glucose (Gadir *et al.*, 2011), these transcripts have decreased association with the mitochondria. It is hypothesized that Puf3p shuttles mRNA targets to the mitochondrial outer membrane surface, where they are locally translated and imported into the mitochondria. This hypothesis is supported by physical interactions between Puf3p and Mdm12p, a mitochondrial outer membrane protein

(Garcia-Rodriguez *et al.*, 2007). Tom20p, a component of the translocase of the mitochondrial outer membrane complex, is also required for mitochondrial localization of Puf3p target mRNAs (Eliyahu *et al.*, 2010). Our results demonstrating that Puf3p physically associates with its target mRNA in both dextrose and galactose conditions further supports the shuttling hypothesis (Figure 2.2). The following model accounts for the dual condition-specific functions of Puf3p. In dextrose conditions, yeast cells can readily perform fermentation, so mitochondria are not required and are in low abundance. To limit mitochondria, expression of mitochondrial proteins is downregulated. One mechanism of such repression involves Puf3p binding to mRNAs such as *COX17*, *TUF1* and *CYT2* and acting to mediate rapid degradation of the transcripts, presumably by recruiting deadenylase and decapping factors. In ethanol, galactose and raffinose conditions when yeast require additional metabolism via the mitochondria, Puf3p's ability to stimulate decay is turned off, but its ability to bind its target mRNAs remains. With the bound mRNA now stabilized, Puf3p can increasingly shuttle the mRNA to the mitochondria for translation and import, though mRNAs could also be shuttled in dextrose conditions before degradation. This role of Puf3p in mRNA localization explains why Puf3p is not downregulated in nonfermenting conditions. Posttranslational modifications such as phosphorylation of either Puf3p or Puf3p binding partners may serve as the molecular switch that inhibits the decay activity of Puf3p. Future work will elucidate the mechanism of these activity changes.

RESULTS

Deadenylation of Puf3p regulated mRNAs is inhibited in galactose conditions

All of the experiments in this section were performed together with Anthony Fischer.

Our previous work began to evaluate the mechanism by which Puf proteins derepress target mRNAs during stress conditions such as respiration (growth in galactose). Our work with Puf3p demonstrated that altered Puf3p activity is not a result of changes in expression, localization or mRNA binding (Miller *et al.*, 2014). In fact, mRNA binding by Puf3p was increased two fold in the inactivating condition galactose (Miller *et al.*, 2014). Furthermore, I evaluated Puf1p, Puf4p and Puf5p using the same criteria as Puf3p and determined that only Puf5p showed a significant reduction in target mRNA binding during stress (Russo and Olivas, see chapter 3). Thus, one mechanism by which Puf5p alters its activity on target mRNAs is through disrupted mRNA binding, as is the case with human Pumilio (Kedde *et al.*, 2010). The mechanism by which Puf1p, Puf3p and Puf4p inactivate decay of target mRNAs in stress conditions remains elusive. To understand the mechanism of Puf3p inactivation, we evaluated which step of decay is being affected by stress. We performed transcriptional pulse-chase experiments to determine if deadenylation is inhibited by galactose conditions. Utilizing our *COX17* reporter mRNA under the control of the inducible/repressible GAL UAS (upstream activating sequence) we were able to create a pulse of newly transcribed *COX17* mRNA, then monitor deadenylation after transcriptional repression. Transcription was repressed by shifting the culture to a non-permissive temperature of 37°C that inactivates RNA polymerase II (*rpb1-1* allele) in this strain. Accomplishing

repression in this manner allowed for deadenylation analysis in both dextrose and galactose conditions as the cells were either maintained in galactose after transcriptional induction or placed in dextrose after transcriptional induction. Utilizing temperature sensitive repression allows for inactivation of transcription regardless of the GAL UAS in the construct. (Russo and Olivas, see chapter 3). As shown in figure 2.3A, deadenylation proceeds rapidly in dextrose, with removal of the poly-adenosine tail within 4-6 minutes (as noted by the smear of products in the lanes) and complete degradation of the mRNA within 6-8 minutes (Figure 2.3A), which is in agreement with previous transcriptional shut-off data for *COX17* mRNA (Olivas and Parker, 2000; Jackson *et al.*, 2004; Houshmandi and Olivas, 2005; Miller *et al.*, 2014). In contrast, in galactose conditions deadenylation is severely inhibited, as mRNAs with at least a partial poly-A tail persist through 40 minutes (Figure 2.3B). These data indicate that carbon-source dependent inactivation of Puf proteins works through the deadenylation step of mRNA decay.

Binding of Pop2p to Puf3p is reduced in galactose

A possible mechanism by which Puf3p is inactivated for mRNA decay stimulation in stress conditions could be differential binding to decay factors. Previous *in vitro* studies have shown that Puf proteins bind many of the core mRNA decay factors including Dcp1p, Dhh1p, Ccr4p and Pop2p (Goldstrohm *et al.*, 2006; Hook *et al.*, 2007; Lee *et al.*, 2010; Quenault *et al.*, 2011). To examine these interactions under stress conditions we performed co-immunoprecipitation (co-ip) studies in dextrose and galactose conditions. Utilizing our *puf3Δ* strain, we individually inserted a C-terminal

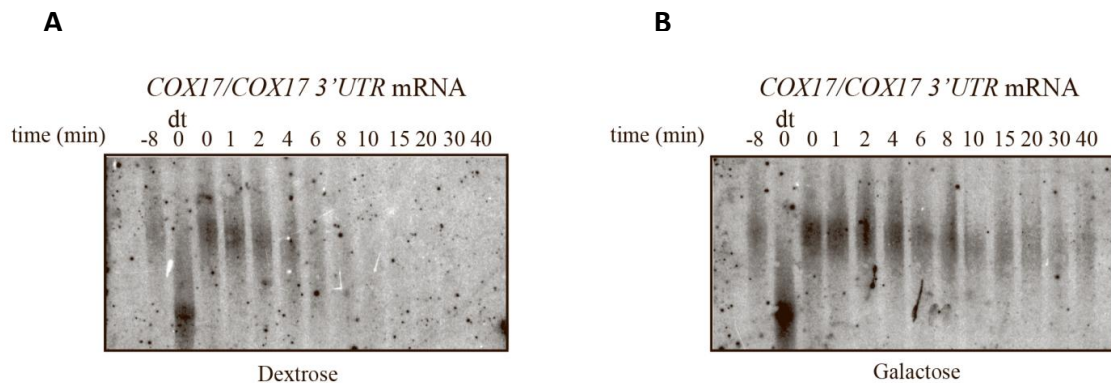


Figure 2.3. Deadenylation rate of *COX17* mRNA during growth in Dextrose or Galactose.

Shown are Northern blot analyses of transcriptional pulse-chase experiments examining decay of *COX17* mRNA during growth in either Dextrose or Galactose. Minutes following transcriptional repression are indicated *above* each blot. The -8 lane in each blot corresponds to background levels of RNA expression before galactose induction of *COX17* RNA transcription. The 0dt lanes in A and B correspond to RNA from the 0-min time point in which the poly(A) tail was removed by RNase H cleavage with oligo(dT). The sugar used in the growth of the cells is indicated below each blot.

Myc-tag into the endogenous genes encoding components of the decay machinery including Dcp1p, Dhh1p, Ccr4p and Pop2p. This resulted in four new strains, each with a single Myc-tagged gene. We then expressed our FLAG-tagged Puf3 repeat domain (RD) construct in each strain. Co-ips were performed to capture Puf3RD from cells grown in either dextrose or galactose conditions, then Myc-tagged factors that co-purified with Puf3RD were evaluated by Western blot. Puf3RD co-purified with Dcp1p, Dhh1p and Ccr4p in both dextrose and galactose to equal levels; however, Pop2p binding was significantly decreased in galactose as compared to dextrose conditions (Figure 2.4, left panel). As a control, Puf3RD was shown to equally purify from both conditions (Figure 2.4, right panel). Furthermore, in galactose Puf3RD increases binding to a smaller form of Pop2p. This smaller form of Pop2p has been previously observed in stationary cells grown in dextrose as well as galactose growth conditions (Norbeck, 2008). These two species of Pop2p have been thoroughly evaluated by cycloheximide (inhibitor of protein synthesis) treatment to ensure that the smaller species is not simply a degradation product (Norbeck, 2008). Thus, a decrease in binding to full-length Pop2p by Puf3RD and an increase in binding to a truncated Pop2p in galactose conditions is likely contributing to Puf3p inactivation.

Pop2p acts as a bridging molecule for Puf3RD interactions with Dcp1p, Dhh1p and Ccr4p *in vivo*

Using *in vitro* binding assays, previous groups have implicated Pop2p as a bridging molecule responsible for interactions of Puf4p and Puf5p with other components of the decay machinery (Goldstrohm *et al.*, 2006; Hook *et al.*, 2007). We

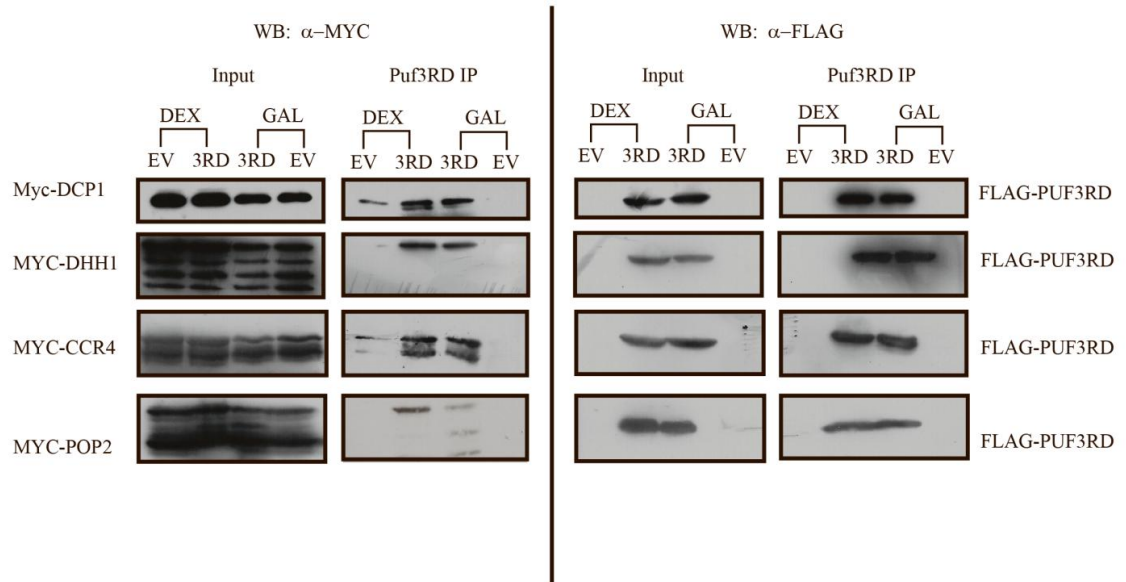


Figure 2.4. Interactions between Puf3p and Pop2p are reduced during growth in galactose. Western blot analysis of co-immunoprecipitation assays performed with FLAG-Puf3RDp in dextrose or galactose conditions. Myc-DCP1, Myc-DHH1, Myc-CCR4 and Myc-POP2 were co-immunoprecipitated with Puf3RDp or empty vector (EV) from dextrose or galactose conditions. Myc-tagged decay factors that co-purified with Puf3RDp were detected using anti-Myc antibody (left panel). As a control, FLAG-Puf3RDp was detected using anti-FLAG antibody to determine equal purification between conditions (right panel). Experiments were performed in triplicate with similar results observed.

provide further support for this hypothesis for Puf3RD binding *in vivo*. Utilizing our *puf3Δ* strain, we created a *puf3Δ/Pop2Δ* double deletion strain. We then endogenously Myc-tagged Dcp1p, Dhh1p or Ccr4p in this strain to create three new strains. Co-ip experiments were performed to capture Puf3RD and eluates were evaluated by Western blot to ascertain if binding to cofactors was dependent on Pop2p. Puf3RD purified equally from both wild-type and *pop2Δ* strains (Figure 2.5, right panel). However, Puf3RD interactions with Dcp1p, Dhh1p and Ccr4p were all disrupted in the absence of Pop2p, supporting the hypothesis that Pop2p acts a bridge between Puf proteins and other decay machinery factors (Figure 2.5, left panel).

Yak1p kinase inactivates Puf proteins in galactose

We next sought to identify the signal for Puf3p inactivation in galactose conditions. Yak1p kinase rapidly phosphorylates (within 2 minutes) Pop2p at Thr97 under glucose deprivation, and this modification is removed within 1 minute upon the addition of glucose (Moriya *et al.*, 2001). Pufp condition-specific regulation of mRNA decay is also rapid, as switching carbon sources can activate or inactivate Pufs within 2 minutes (Miller *et al.*, 2014) (Russo and Olivas, submitted). To determine if Yak1p is responsible for inactivation of Puf3p in galactose, we knocked out *YAK1* in our temperature sensitive strain used to perform RNA decay analysis. Such transcriptional shut-off assays were performed in both dextrose and galactose in the wild-type strain and the *yak1Δ* strain. In contrast to the wild-type strain, *COX17* mRNA was rapidly degraded in galactose in the absence of Yak1p, supporting the hypothesis that the presence of Yak1p is involved in inactivation of Puf3p (Figure 2.6). This data supports

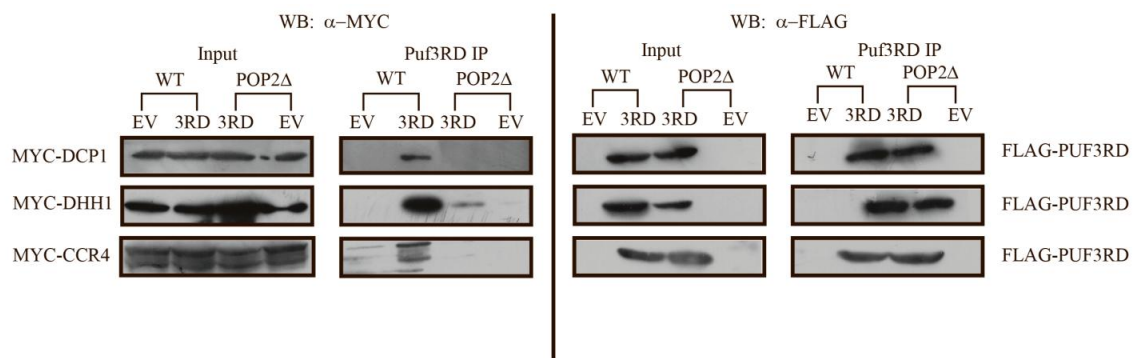


Figure 2.5. Interactions between Puf3p and Dcp1p, Dhh1p and Ccr4p are bridged by Pop2p. Western blot analysis of co-immunoprecipitation assays performed with FLAG-Puf3RDp in a *PUF3 Δ /POP2 Δ* strain. Myc-DCP1, Myc-DHH1, and Myc-CCR4 were co-immunoprecipitated with Puf3RDp or empty vector (EV). Myc-tagged decay factors that co-purified with Puf3RDp were detected using anti-Myc antibody (left panel). As a control, FLAG-Puf3RDp was detected using anti-FLAG antibody to determine equal purification between conditions (right panel). Experiments were performed in triplicate.

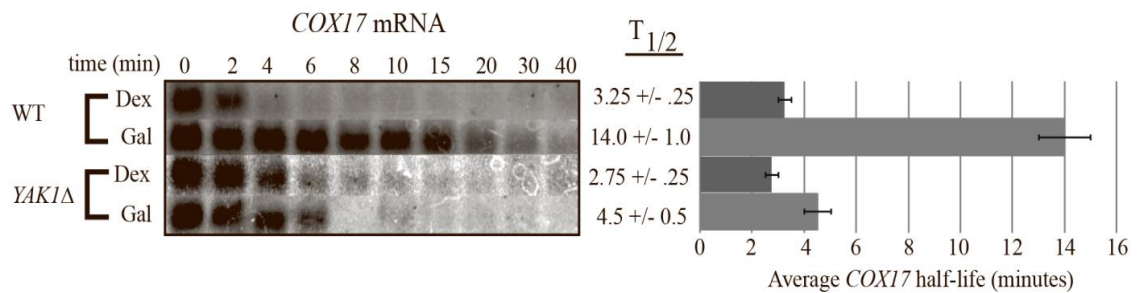


Figure 2.6. Condition-specific activity of Puf3p is dependent on Yak1p. Decay of *COX17* mRNA in wild-type or *yak1*Δ strains during growth in dextrose or galactose is shown. Representative Northern blots are presented in the left panel, with average half-life (T_{1/2}) listed to the right of each blot. Graphical representation of average half-life is presented in the right panel. Minutes following transcriptional shut-off are indicated above blots and along the X-axis of the graph. The error for each T_{1/2} is the SEM (n≥3).

the hypothesis that Pop2p phosphorylation by Yak1p inhibits Puf3p activity, likely through disruption of Puf3p binding to Pop2p.

Puf3RD-R7A cannot bind full length Pop2p in dextrose conditions

Previous work from our lab described a Puf3RD mutant, *Puf3RD-R7A*, which has four amino acids deleted from the outer surface of the Puf repeat 7 (Houshmandi and Olivas, 2005). This mutant is able to bind *COX17* mRNA; however, it is not able to stimulate its decay (Houshmandi and Olivas, 2005). We sought to further characterize the mechanism by which this mutant cannot stimulate decay. Based on our discovery that Pop2p is differentially bound to Puf3p in inactivating conditions, we performed similar co-ips with our *Puf3RD-R7A* mutant. Again, wild-type Puf3RD preferentially bound full length Pop2p in dextrose; however, *Puf3RD-7A* nearly exclusively binds the smaller form of Pop2p (Figure 2.7, left panel). In fact, the full length Pop2p is often undetectable even in the original input when Puf3RD-R7A is expressed, suggesting that the interaction between the full length Pop2p and Puf3p may stabilize full length Pop2p (Figure 2.7, left panel). Dcp1p, Dhh1p and Ccr4p were all able to bind both wild-type and the mutant equally (Figure 2.7, left panel). As a control, Western blot analysis using α -FLAG antibody illustrates equal purification levels of Puf3RD and *Puf3RD-R7A* (Figure 2.7, right panel). We hypothesize that the lower form of Pop2p bound to *Puf3RD-7A* is not functional to mediate decay, and is therefore responsible for the previously observed stabilization of *COX17* mRNA (Houshmandi and Olivas, 2005).

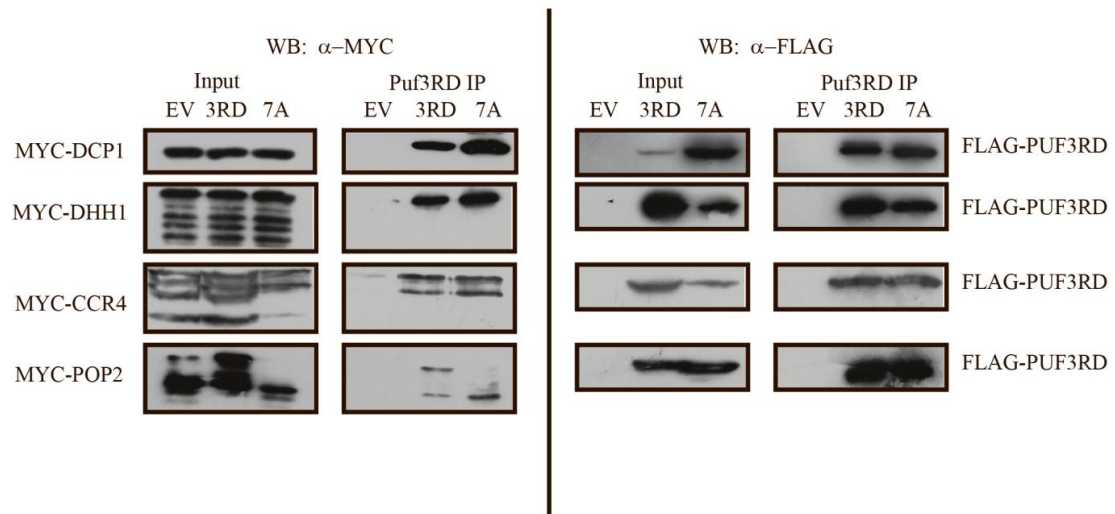


Figure 2.7. Mutant *Puf3RD-R7A* binds a truncated version of Pop2p. Western blot analysis of co-immunoprecipitation assays performed with FLAG-Puf3RDp or *FLAG-Puf3RD-R7A* in dextrose conditions. Myc-DCP1, Myc-DHH1, Myc-CCR4 and Myc-POP2 were co-immunoprecipitated with Puf3RDp, *Puf3RD-R7A* (7A) or empty vector (EV) from dextrose conditions. Myc-tagged decay factors that co-purified with Puf3RDp or *Puf3RD-R7A* were detected using anti-Myc antibody (left panel). As a control, FLAG-Puf3RDp was detected using anti-FLAG antibody to determine equal purification between conditions (right panel). Experiments were performed in triplicate.

The lower form of Pop2p is an N-terminal truncation

Previous studies have also observed two forms of Pop2p that exist in *S. cerevisiae*, suggesting biological relevance (Norbeck, 2008). We have shown that the truncated form of Pop2p may be a dominant negative form that cannot stimulate decay of Puf3p target mRNAs. To further characterize the truncated form of Pop2p, we purified our FLAG-Puf3RD and FLAG-Puf3RD-R7A in dextrose conditions. These two proteins showed the strongest differential binding of the two forms of Pop2p. We then coomassie stained and excised the two Pop2p bands using a western blot of the same sample for reference. The two species were subjected to mass spectrometry. The results showed that peptide fragments consisting of the first 140 residues were not detectable in the lower Pop2p band, supporting the hypothesis that the smaller Pop2p is an N-terminal truncation.

DISCUSSION

The biological role of the Puf family of RNA binding proteins in eukaryotes continues to expand. Recently, Puf proteins in yeast have been implicated in stress response (Miller *et al.*, 2014) (Russo and Olivas, submitted). In response to respiration, Puf1p, Puf3p, Puf4p and Puf5p are inactivated and target mRNAs are derepressed allowing translation of many stress response genes including *YHB1* (Miller *et al.*, 2014) (Russo and Olivas, submitted). The inactivation of Puf proteins is rapid and can be observed within 2 minutes upon carbon source change from dextrose to galactose (Miller *et al.*, 2014) (Russo and Olivas, submitted). Thorough evaluation of differential expression, localization and mRNA binding of Puf proteins in yeast revealed only Puf5p

exhibits reduced target mRNA binding. Thus, the mechanism of inactivation of Puf proteins is still unrefined (Miller *et al.*, 2014) (Russo and Olivas, submitted). Based on the rapid activation/inactivation switch for Puf proteins, we hypothesize that the signal responsible is likely post-translational.

Puf proteins provide target specificity by binding to specific mRNAs and direct interactions with Pop2p bridge interactions with decay machinery including Dcp1p, Dhh1p and Ccr4p (Goldstrohm *et al.*, 2006; Hook *et al.*, 2007; Lee *et al.*, 2010; Miller and Olivas, 2011; Quenault *et al.*, 2011). Furthermore, Pop2p is phosphorylated by Yak1p in response to glucose deprivation and this modification is rapidly removed upon glucose addition (Moriya *et al.*, 2001). Therefore, it is plausible that the interaction between Pop2p and Puf proteins acts as a switch for repression of target mRNAs. We discovered that the deadenylation step of decay is inhibited in respiration conditions and Puf3p binding to Pop2p is reduced in respiration conditions compared to fermentation conditions, supporting the hypothesis that interactions between Pop2p and Puf3p control repressive activity. Perhaps phosphorylation of Pop2p upon glucose deprivation disrupts specific interactions with Puf3p; however, interactions with other proteins are unperturbed. This hypothesis is further supported by transcriptional shut-off data in a *YAK1Δ* strain grown in respiration conditions where repression was maintained despite stress conditions. In addition, Puf3p interaction with a smaller form of Pop2p is increased in respiration conditions and a mutant form of Puf3RD with a four amino acid deletion of an outer surface binding loop nearly exclusively binds the smaller form. In fact, the full length Pop2p levels in lysate were often reduced in

respiration conditions and nearly non-detectable during Puf3RD-R7A expression. These data provide strong support for the smaller version of Pop2p having a biological role as a dominant negative inhibitor of repression. Further support comes from cycloheximide treatment of cells followed by a time course evaluation of Pop2p degradation which showed that the two species are present at steady state and are stable throughout the 30 minute time course (Norbeck, 2008). We have begun to characterize the smaller version of Pop2p by mass spectrometry and the limited coverage obtained suggests it is an N-terminal truncation. Further research will biochemically characterize the truncated form of Pop2p.

We propose a model for carbon source-mediated Pufp inactivation. In response to glucose deprivation Pop2p is phosphorylated by Yak1p. The modified version of Pop2p has reduced binding to Puf proteins; however, binding to other protein partners is maintained. An increase in binding of the truncated Pop2p to Puf proteins results in decreased repression of the mRNA target. This allows an increase in protein products derived from Puf target mRNAs to combat stress conditions presented to the cell.

MATERIAL AND METHODS

Yeast strains

Yeast strains used in this study are listed in table 1.2. For construction of yWO270, yWO271 and yWO272 TRP1 was replaced with KanMX by homologous recombination using pWO182, a marker swap vector (Voth *et al.*, 2003). For

construction of Myc-tagged cofactor strains, primers containing sequence homologous to the C-terminus of each protein and a common Myc region were used to amplify Myc₉-TRP1 from pCH985 (C.F.J. Hardy). The Myc₉ inserts with homologous ends to the gene of choice were transformed into yWO18, yWO270, yWO271, yWO272 and selected on –trp media. Colonies were verified for C-terminus Myc-tag insertion by PCR verification using genomic DNA and Western blot analysis.

Plasmids

Plasmids used in this study are listed in table 2.2. For construction of mutant POP2 plasmids, site-directed mutagenesis was used in accordance with manufacturers protocol (Stratagene). All constructs were verified by sequencing. For construction of the HIS-reporter plasmids

In vivo decay analysis

Decay of reporter mRNAs was monitored in strains harboring the temperature-sensitive *rpb1-1* RNA polymerase II allele, in which transcription is rapidly halted following a shift from 24°C to 37°C. All yeast transformations were performed by LiOAc high-efficiency transformation (Gietz *et al.*, 1995).

Transcriptional shut-offs were performed as described (Caponigro *et al.*, 1993) in the yeast strain yWO7 (*rpb1-1*), yWO294(*rpb1-1, pop2Δ*) and yWO292(*rpb1-1, yak1Δ*). pWO224, pWO225 or empty vector was transformed into yWO290. Strains were grown as 200 mL cultures in Yeast extract peptone (YEP) containing 2% dextrose or 2% galactose and 100mg/mL Zeocin (excluding yWO7 and yWO292) at 24°C to an OD₆₀₀ of 0.4. The culture was harvested and resuspended in 20 mL YEP containing 8% dextrose

or galactose at 37°C, effectively shutting off transcription by the temperature-sensitive inactivation of RNA pol II. Time course samples were taken over a 40 minute time period at 37°C. Total RNA was isolated from yeast samples as described (Caponigro *et al.*, 1993), followed by northern blot analysis (Biobond plus nylon membrane-Sigma). Northern blots were probed with ³²P-end-labeled oWO2, to detect *COX17* mRNA. Loading was normalized using *scRI* RNA, a constitutively expressed RNA polymerase III transcript, and all imaging and quantification of half-lives were determined by ImageQuant software (Molecular Dynamics).

Transcriptional pulse chase experiments were performed essentially as described (Decker and Parker, 1993) on strains yWO52(*cox17Δ*, *rpb1-1*) and yWO269(*yhb1Δ*, *rpb1-1*). Condition regulated expression of *COX17* or *YHB1* RNA was accomplished by transformation of yWO52 or yWO269 with pWO5(Beers *et al.*, 1997) or pWO128(Russo and Olivas), respectively in which the expression of the RNA is under the control of the *GAL* UAS. Cultures were grown in 2% raffinose, which does not induce transcription of the RNA, to an OD600 of 0.4 or 1.0 for *COX17* or *YHB1*, respectively. Cultures were then incubated with galactose to induce transcription of this RNA for 8 min to create a pulse of newly transcribed RNAs. Finally, pre-warmed dextrose or galactose was added to the media raising the temperature to 37°C effectively repressing transcription of the RNAs. Poly(A) tail lengths were monitored using RNaseH reactions as previously described (Muhlrad and Parker, 1992) with an oligo complementary to a sequence just upstream of the *COX17* or *YHB1* stop codon (oWO1 or oWO894, respectively). Total RNA was separated on 6% denaturing polyacrylamide gels at 300V for 6 h, then transferred

to nylon membrane for probing with a radiolabeled oligo to *COX17* or *YHB1* (oWO2 or oWO895).

Puf protein co-immunoprecipitation analysis

Condition-specific Puf3 repeat domain (RD) co-immunoprecipitation (co-ip) studies were performed as follows. pWO16 (Puf3RD) or pWO15 (empty vector) were transformed into yWO187 (Myc-CCR4), yWO188 (Myc-DCP1), yWO189 (Myc-DHH1) or yWO191 (Myc-POP2). Cells were grown as 200mL cultures of selective minimal media supplemented with 2% dextrose or 2% galactose to an OD600 of 0.4. Cells were harvested and frozen in a 2mL eppendorf tube at -80°C until co-ip was performed. Cell pellets were resuspended in 500μL of co-ip binding buffer containing 50mM Tris-HCl pH 7.5, 50mM NaCl, 2mM MgCl₂, 0.1% NP-40 and 10% glycerol. Immediately before resuspension, 2-mercaptoethanol (0.01%v/v), 1 protease inhibitor tablet per 10mL (Roche) and RNASE A (40μg/mL) were added. 100μL of FLAG affinity resin (Sigma) per co-ip was equilibrated in 1mL of co-ip binding buffer supplemented with 1mM PMSF and 1%BSA for ≥ 1 hour at 4°C. Resuspended cells were subjected to manual lysing using glass beads and vortexing for 1 minute followed by 1 minute on ice (5X). Lysate was quantitated using Bradford reagent (Bio-Rad) and equal amounts of total protein were loaded onto the FLAG resin. A portion of the lysate was saved for input analysis. The lysate was allowed to bind to the FLAG resin for 1 hour at 4°C on an end over end rocker. After 1 hour, the beads were spun down and lysate was removed. The beads were then subjected to 3, 5 minute washes with co-ip wash buffer consisting of 50mM Tris-HCl pH 7.5, 150mM NaCl, 2mM MgCl₂, 0.5% NP-40, and 1mM PMSF. After the final

wash, FLAG-Puf3RD was eluted by competition using 3X FLAG peptide (Sigma). 4 μ L of a 5mg/mL 3X FLAG peptide solution was added to 96 μ L of wash buffer for a final volume of 100 μ L per co-ip sample. Elution was allowed to proceed for 20 minutes at 4°C with end over end rocking. The beads were separated out by pipetting the co-ip/bead mixture into a Spin-X column and centrifuging in a table top centrifuge for 1 minute at 4°C. The eluted protein is captured in the flow-through. Equal volumes of lysate and co-ip were loaded onto a 7.5% SDS-PAGE gel for separation and then subjected to western blotting. Often, 2 identical gels were run simultaneously for probing with α -FLAG (Sigma) for detection of the Puf3RD and α -MYC for detection of co-factors.

Condition-specific Puf1, Puf4 and Puf5 co-ips were performed similar to the Puf3RD co-ips with the following modifications. Endogenously TAP-tagged Puf1p, Puf4p, Puf5p strains containing an endogenously Myc-tagged POP2 were grown in a 100mL culture of YEP in the presence of 2% dextrose or 2% galactose to an OD600 of 1.0. The lysate was allowed to bind to the IGG sepharose 6 fastflow (GE healthcare) or Sepharose 6B resin for 1 hour at 4°C on an end over end rocker. TAP-Puf proteins were eluted from the beads using 4 μ L of a 5mg/mL Protein A solution added to 96 μ L of wash buffer for a final volume of 100 μ L per co-ip sample. During blocking of the membranes for western blotting, a non-specific antibody (α -LRRK2, human) was used to block Protein A signal.

Puf3RD co-ip was performed in the absence of *POP2*. The co-ip experiments were performed as described above with the following modifications. yWO285,

yWO290 and yWO291 were transformed with pWO15 (empty vector) or pWO15 (Puf3RD).

Puf3RD co-ip was performed in the presence of POP2 WT (pWO135), *pop2* T97A (pWO224), *pop2* T97D (pWO225) in yWO290 (*puf3Δpop2Δ*). Cultures were grown as described for the Puf3RD co-ip with the addition of 100mg/mL Zeocin. Plasmid encoded Pop2p was detected using α -His antibody during Western blotting.

Puf3RD R7A loop mutant co-ip was performed as described for Puf3RD. pWO86 (Puf3RD R7A) was transformed into yWO187 (Myc-CCR4), yWO188 (Myc-DCP1), yWO189 (Myc-DHH1) or yWO191 (Myc-POP2).

Repression assays

The *HIS-COX17* 3' UTR reporter gene contains the MET25 promoter driving the *HIS3* coding region and the *COX17* 3' UTR downstream. The *cox17* mt has both canonical PREs mutated from UGUA to ACAC by site-directed mutagenesis. yWO211 (WT), yWO212 (*pop2Δ*) and yWO213 (*ccr4Δ*) were transformed with each reporter RNA and Puf3FL (pWO226). Additionally, yWO212 was transformed with pWO135, pWO224 or pWO225. For growth assay, colonies were isolated and grown to an OD600 of 0.4-0.6 at 30 °C and plated on minimal medium with or without Histidine. The *HIS3* competitive inhibitor 3-aminotriazole was added to increase stringency where indicated.

Mass spectrum analysis

For mass spectrum analysis of two isoforms of POP2p, 4 liter cultures were grown using yWO191 transformed with pWO16(isolation of full length Pop2p) or pWO86(isolation of truncated Pop2p). Co-ip was performed as described above after appropriate scaling for

the larger culture volume. Total IP was run on a SDS-PAGE gel and coomassie stained for band visualization. The appropriate bands were excised from the gel and frozen. A small portion of the IP was saved for western blot analysis and as a reference for excision of POP2p bands. The gel bands were sent to the University of Missouri-Colombia for analysis.

Table 1.2. Oligos used in this chapter

oWO	Sequence	Use
1	gccataacccttcatgcactc	<i>COX17</i> cutter
2	ggttgctggcagactgtcag	<i>COX17</i> probe
21	gtctagccgcgaggaagg	<i>7Sr</i> RNA probe
834	ccagtcattccaaacggaattcggtccttaccgctgctcgctccacatacgatttaggtgacac	<i>POP2::NAT</i> up
835	gaccgctaactatacggaataacgcatcaatctcctgtggaagagtgaatacgaactcacta tagggag	<i>POP2::NAT</i> down
875	cagcagccatttaatataggagatcctgtttcggtggctagtttg	<i>POP2</i> T97D up
876	aaactagccaccgaaacaggatctcctatattaaatggctgctg	<i>POP2</i> T97D down
877	cagcagccatttaatataggagctcctgtttcggtggctagtttg	<i>POP2</i> T97A up
878	caaactagccaccgaaacaggagctcctatattaaatggctgctg	<i>POP2</i> T97A down
879	caatctatgaatgtacaaccg	<i>POP2</i> sequence primer

Table 2.2. Plasmids Used in This Study

Plasmid	Description	Marker(s)	Source
pWO5	pST 30 pGAL-COX17	Amp ^R , LEU2	Olivas and Parker, 2000
pWO15	pG-1 derivative/FLAG tag	Amp ^R , URA3, TRP1	Schena and Yamamoto, 1988
pWO16	FLAG-PUF3RD	Amp ^R , URA3, TRP1	Olivas and Russo, 2014
pWO135	pACG NT POP2	Zeo ^R	Goldstrohm et al. 2007
pWO166	TRP1→URA3 marker swap	Amp ^R	ATCC 87550
pWO224	pACG NT POP2 T97A	Zeo ^R	This study
pWO225	pACG NT POP2 T97D	Zeo ^R	This study
pWO226	pG-1 PUF3 TRP1→URA3	Amp ^R , URA3	This study
pWO227	pMET-HIS3-COX17 3' UTR	Amp ^R , LEU2	This study
pWO228	pMET-HIS3-COX17 3' UTR mt	Amp ^R , LEU2	This study

Table 3.2. Strains used in this study

Description	Strain	Genotype	Source
Wild-type	yWO7	MAT α , <i>leu2-3,ura3-52, rpb1-1</i>	Caponigro <i>et al.</i> 1993
<i>puf3</i> Δ	yWO18	MAT α , <i>trp1, ura3-52, leu2-3, his4-539, cup1::LEU2(PM), PUF3::NEO</i>	Olivas and Parker, 2000
<i>cox17</i> Δ	yWO52	MAT α , <i>trp1, leu2-3, ura3-52, his3-1, his4-539, rpb1-1, COX17::TRP1</i>	Wendy Olivas
Myc-CCR4	yWO187	MAT α , <i>trp1, ura3-52, leu2-3, his4-539, cup1::LEU2(PM), PUF3::NEO, Myc-CCR4, TRP1</i>	This study
Myc-DCP1	yWO188	MAT α , <i>trp1, ura3-52, leu2-3, his4-539, cup1::LEU2(PM), PUF3::NEO, Myc-DCP1, TRP1</i>	This study
Myc-DHH1	yWO189	MAT α , <i>trp1, ura3-52, leu2-3, his4-539, cup1::LEU2(PM), PUF3::NEO Myc-DHH1, TRP1</i>	This study
Myc-POP2	yWO191	MAT α , <i>trp1, ura3-52, leu2-3, his4-539, cup1::LEU2(PM), PUF3::NEO Myc-POP2, TRP1</i>	This study
Myc-DHH1, <i>POP2</i> Δ	yWO285	MAT α , <i>trp1, ura3-52, leu2-3, his4-539, cup1::LEU2(PM), PUF3::NEO, Myc-DHH1, TRP1, POP2::NTC</i>	This study
Myc-DCP1, <i>POP2</i> Δ	yWO290	MAT α , <i>trp1, ura3-52, leu2-3, his4-539, cup1::LEU2(PM), PUF3::NEO, Myc-DCP1, TRP1, POP2::NTC</i>	This study
Myc-CCR4, <i>POP2</i> Δ	yWO291	MAT α , <i>trp1, ura3-52, leu2-3, his4-539, cup1::LEU2(PM), PUF3::NEO, Myc-CCR4, TRP1, POP2::NTC</i>	This study
<i>YAK1</i> Δ	yWO292	MAT α , <i>leu2-3,ura3-52, rpb1-1, YAK1::NTC</i>	This study
<i>POP2</i> Δ	yWO294	MAT α , <i>leu2-3,ura3-52, rpb1-1, POP2::NTC</i>	This study

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**CHAPTER 3: CONDITIONAL REGULATION OF PUF1P, PUF4P AND
PUF5P ACTIVITY ALTERS *YHB1* MRNA STABILITY FOR A RAPID
RESPONSE TO TOXIC NITRIC OXIDE STRESS IN YEAST.**

INTRODUCTION

Cells must rapidly adapt to various types of environmental signals and stresses. Efficient methods to rapidly alter gene expression in response to such signals include the post-transcriptional regulation of mRNA translation and decay rates. Some of the most familiar cases of decay control come from mammalian proto-oncogenes, cytokines, and transcription factors, whose mRNAs are targeted for rapid decay in response to environmental stimuli (Shim and Karin, 2002). Changes in decay rates of specific transcripts are also involved in circadian clock control (Lidder *et al.*, 2005), the cell cycle (Penelova *et al.*, 2005), oxidant stress response by CFTR (Cantin *et al.*, 2006) and differentiation (Jack and Wabl, 1988). The regulatory elements for these changes are often found within the 3' untranslated region (UTR) where regulatory RNA-binding proteins attach and perform repressive functions.

The Puf protein family is a largely conserved class of RNA-binding proteins across eukaryotes (Wickens *et al.*, 2002; Miller and Olivas, 2011). Puf proteins have a diverse set of roles including stem cell maintenance, (Forbes and Lehmann, 1998; Parisi and Lin, 1999; Crittenden *et al.*, 2002; Moore *et al.*, 2003), development and differentiation, (Murata and Wharton, 1995), neuronal plasticity (Menon *et al.*, 2004) and stress response (Foat *et al.*, 2005; Miller *et al.*, 2014). At the molecular level, Puf proteins repress mRNAs by interacting with sequence elements typically located in the 3' UTR of target mRNAs. Once bound to an mRNA, Puf proteins elicit repression either through protein interactions that inhibit cap-binding events of translation initiation, or interactions with mRNA decay machinery to stimulate deadenylation and decapping steps of decay (Goldstrohm *et al.*, 2006; Goldstrohm *et al.*, 2007; Lee *et al.*, 2010; Miller

and Olivas, 2011).

The Puf family is characterized by a conserved RNA-binding domain consisting of eight imperfect repeats of a 36 amino acid sequence, plus short flanking regions. Crystal structure analysis of multiple Puf-mRNA complexes has revealed a primarily modular binding method wherein conserved amino acids within each repeat contact and stack with successive bases along the RNA, though binding specificity and flexibility involves some RNA bases flipping out from the protein binding surface (Edwards *et al.*, 2001; Wang *et al.*, 2001; Wang *et al.*, 2002; Miller *et al.*, 2008; Wang *et al.*, 2009; Zhu *et al.*, 2009). The conserved Puf recognition element (PRE) contains a UGU sequence followed by an AU-rich region, though different Pufs bind specific variants of the AU-rich region (Murata and Wharton, 1995; Wreden *et al.*, 1997; Zamore *et al.*, 1997; Souza *et al.*, 1999; Zamore *et al.*, 1999; Nakahata *et al.*, 2001; Tadauchi *et al.*, 2001; Wang *et al.*, 2001; Wang *et al.*, 2002; Gerber *et al.*, 2004; Jackson *et al.*, 2004).

Saccharomyces cerevisiae contains six Puf proteins (Puf1p-Puf6p). All except Puf2p have been shown to stimulate mRNA decay and/or repress translation via 3'UTR interaction (Olivas and Parker, 2000; Tadauchi *et al.*, 2001; Gu *et al.*, 2004; Goldstrohm *et al.*, 2006; Hook *et al.*, 2007; Ulbricht and Olivas, 2008). The function of several bona fide and putative targets of Puf regulation in yeast relates to stress response (Foat *et al.*, 2005; Garcia-Rodriguez *et al.*, 2007; Miller *et al.*, 2014). In this work we evaluate *YHB1* mRNA as a putative target of Puf-mediated decay stimulation in yeast. *YHB1* was originally identified in a microarray analyzing altered RNA levels in a yeast strain deleted of *PUFs* 1-5 (Olivas & Parker 2000). The *YHB1* 3' UTR contains two overlapping PREs that

can potentially be utilized by multiple Puf proteins, though not simultaneously.

YHB1 encodes the only known defense protein against nitric oxide (NO) stress in *S. cerevisiae* (Liu *et al.*, 2000). To counteract the toxic effects of NO, expression of the flavohemoglobin Yhb1p is induced, which metabolizes NO into nitrate or dinitrogen oxide depending on the aerobic or anaerobic conditions (Foster *et al.*, 2009). Concomitant with its function, Yhb1p is localized to both the cytosol and the mitochondrial matrix (Cassanova *et al.*, 2005). The absence of *YHB1* causes growth inhibition when cells are treated with the aerobic NO donor, Deta-NO (Liu *et al.*, 2000; Foster *et al.*, 2009). The regulation of *YHB1* expression occurs at both transcriptional and post-transcriptional levels, and by multiple environmental signals. Transcriptional upregulation occurs in response to both NO exposure and the available sugar source through independent transcription factor mechanisms, and Yhb1p levels have been shown to immediately decrease upon the addition of glucose (Zhu *et al.*, 2006). Within yeast cells, mitochondrial cytochrome oxidase produces NO, and the amount of NO production is dependent on the amount of respiration in the cell (Li *et al.*, 2011). The addition of glucose limits the need for respiration in yeast cells, which limits NO production and the need for Yhb1p. In addition to transcriptional control, regulation of mRNA decay and/or translation rates offers a rapid response to such environmental stresses.

Our previous work demonstrated that Puf3p stimulates mRNA decay of nuclear-encoded mitochondrial transcripts in fermentative growth conditions (in glucose) when mitochondrial respiration is not required. However, Puf3p activity is inhibited in

ethanol, galactose and raffinose conditions that use mitochondria, thereby stabilizing the mitochondrial transcripts for increased translation. The response to carbon source by Puf3p is rapid and not due to altered Puf3p expression (Miller *et al.*, 2014). The ability of other Pufs to respond to stress by altering regulatory capacity for target mRNAs is unknown.

To further our understanding of condition-specific mRNA decay stimulation by Puf proteins, we evaluated the *YHB1* 3'UTR for the ability to confer Puf regulation. We determined that Puf1p, Puf4p and Puf5p all play a role in stimulating *YHB1* mRNA decay through binding two overlapping PREs in the *YHB1* 3' UTR. This Puf-mediated decay stimulation is inhibited under stress conditions when Yhp1p production is required, with reduced Puf5p binding activity contributing to decay inhibition. However, overexpression of Pufs can override such inactivation, and Puf overexpression during NO exposure can destabilize *YHB1* mRNA, resulting in decreased cell growth. Together, this work advances our understanding of the multifaceted mechanisms of controlling *YHB1* mRNA stability and cell fitness in rapid response to stress.

RESULTS

All of the results presented in this section were complete by me.

***YHB1* mRNA is destabilized by multiple Puf proteins**

The yeast *YHB1* transcript was first identified as a potential target of Puf protein-mediated decay stimulation based on its increased expression in a Puf deletion strain ($\Delta puf1-5$) versus a wild-type strain (Olivas and Parker, 2000). *YHB1* mRNA contains two overlapping candidate PREs located in its 3' UTR (Figure 3.1A). To evaluate *YHB1* mRNA for control by Puf proteins, decay analysis was performed in a wild-type *PUF* strain (WT),

strains deleted of individual *PUFs* 1-5, or a quintuple mutant ($\Delta puf1-5$). To ascertain the direct role of Puf protein regulation through the *YHB1* 3' UTR, we created a reporter vector in which the 3' UTR of *YHB1* is cloned downstream of the stable coding region of *PGK1*. Prior studies have shown that the 3' UTRs of Puf-regulated targets are sufficient to confer rapid decay to otherwise stable mRNAs (Jackson *et al.*, 2004). Half-lives were determined following transcriptional repression by two simultaneous methods. First, strains containing the temperature-sensitive RNA polymerase II allele (*rpb1-1*) were used to inhibit transcription by shifting to a non-permissive temperature (Herrick *et al.*, 1990). Second, promoter-specific transcriptional repression of the *PGK1/YHB1* 3'UTR reporter under the control of the *GAL* UAS was achieved by shifting from galactose to dextrose in the media (Decker and Parker, 1993).

In WT yeast the *PGK1/YHB1* 3' UTR reporter mRNA decayed with a half-life of 10.7 ± 1.3 min (Figure 3.1B). In the *puf2* Δ and *puf3* Δ strains the reporter half-life was similar to that of WT; however, in the *puf1* Δ , *puf4* Δ and *puf5* Δ strains the reporter half-life was significantly extended by 1.5 to 2 fold, suggesting Pufs 1, 4 and 5 all contribute to the destabilization of *YHB1* mRNA (Figure 3.1B). To evaluate the full extent of Puf destabilization on our reporter, we determined the half-life in the $\Delta puf1-5$ strain to be 24.3 ± 2.2 min (Figure 3.1B). Both the *puf4* Δ and *puf5* Δ strains showed shorter half-lives compared to the $\Delta puf1-5$, suggesting that Puf4p and Puf5p do not act redundantly, and the level of active Pufs may be limiting the decay rate of *YHB1* mRNA. These data also indicate that Puf4p and Puf5p contribute equally to decay stimulation of *YHB1* mRNA, while Puf1p plays less of a role.

A single flexible binding site is required for Puf1p, Puf4p and Puf5p destabilization of *YHB1* mRNA.

Previously identified targets of multiple Puf regulation such as *HO*, *HXK1* and *TIF1* mRNAs contain multiple, non-overlapping PREs in their 3' UTRs (Hook *et al.*, 2007; Ulbricht and Olivas, 2008). In contrast, while the *YHB1* mRNA 3' UTR is also regulated by multiple Pufs, it potentially contains two overlapping PREs, such that only one Puf protein could bind this region at one time. To determine if one or both PREs are necessary for Puf-mediated decay, we mutated the conserved UGU element within each PRE, which has been shown to be required for Puf binding (Jackson *et al.*, 2004; Ulbricht and Olivas, 2008). If a particular UGU element is necessary for Puf-mediated decay, its mutation will extend the mRNA's half-life in a wild-type *PUF* strain. To test this hypothesis, we either mutated the first UG in the first PRE while leaving the second PRE intact (site #1 mutant) or mutated the UGU elements in both PREs (site #2 mutant) (Figure 3.2A). We did not make mutations in the second UGU alone because this would also disrupt the first PRE. The site #1 mutant mRNA, *PGK1/yhb1-1*, decayed with a half-life 1.5 fold longer than the WT mRNA, suggesting that disruption of the first PRE inhibits optimal regulation of the reporter, although the second PRE can still function to mediate decay in the absence of the first PRE (Figure 3.2B). The site #2 mutant mRNA, *PGK1/yhb1-2*, decayed with a half-life 2.4 fold longer than the WT mRNA, similar to the half-life in the $\Delta puf1-5$ strain, indicating that all Puf-mediated decay stimulation acts through these overlapping PREs. Unlike other known targets of multiple Puf decay

A.

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UAGGCGUUAAGCCUCGGAACUUCAAUUUUUAUUACUGAUUCAUUUGAUAAUUUCACU
stop
UGUU CGAUUUCACAGUUUGAGAAGUUAUUAUUACGAAAUAAGAAAAUUUUUAAAACAUG
AGACCAUUGC UAAUCUUUUGUUGUUAUACUGUGUUAGAAAUCCUCGUGAAAUCCUUG
UGAAAUCCUAUGUGGAAUAUUUAGAUAGUAAGUAAAGAUGUGUGUAUGUAUUUAUAAGA
site #1 site #2
UGAAUAAGCGCCAGGUGC UAAACAGAGUUCACUUCACGUUAACGCCAGUCCAAUUUU
UGAUUGCAUUC CCGUUUUU

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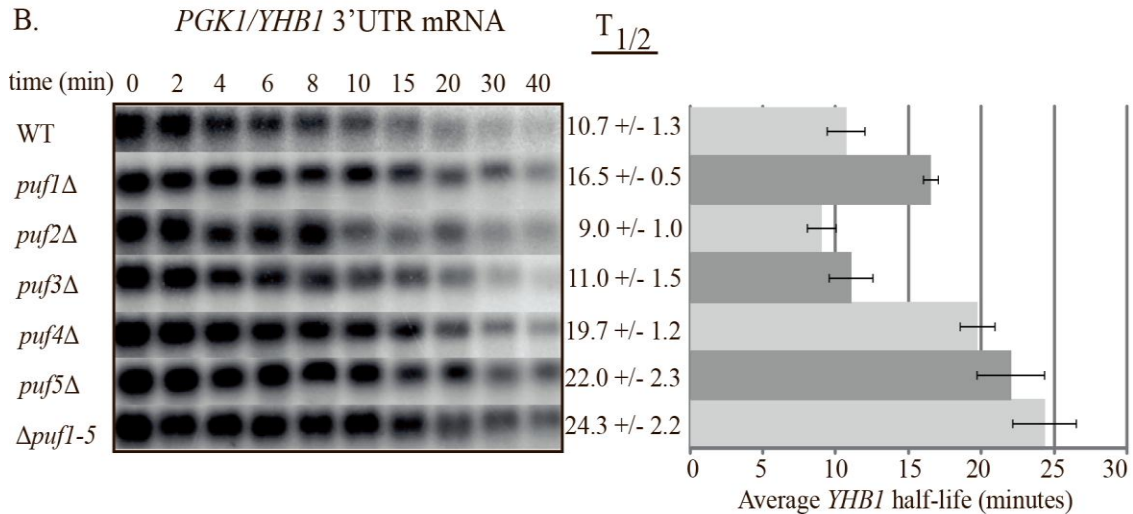


Figure 1.3. Puf1p, Puf4p and Puf5p regulate *YHB1* mRNA stability. (A) Sequence of the *YHB1* 3' UTR. Canonical binding sequences of Puf proteins are in bold. Underlined regions are candidate sites of Puf interaction (Site #1 and Site #2). UGUN sequences are shaded gray. (B) Decay of *PGK1/YHB1* 3' UTR fusion mRNA in wild-type (WT), individual *PUF* deletion, and $\Delta puf1-5$ yeast strains grown to an OD₆₀₀=1.0 in galactose, then transcription repressed at time 0 with addition of dextrose. Representative Northern blots are presented in the left panel, with average half-life ($T_{1/2}$) listed to the right of each blot. Graphical representation of average half-life is presented in the right panel. Minutes following transcriptional shut-off are indicated above blots and along the X-axis of the graph. The error for each $T_{1/2}$ is the SEM ($n \geq 3$).

regulation, *YHB1* mRNA is unique because only one binding site containing overlapping PREs is necessary and sufficient to confer destabilization by three Puf proteins. To evaluate whether differential RNA binding contributes to the differential roles of Puf1p, Puf4p and Puf5p in decay stimulation, as well as the decreased decay stimulation with the mutant sites, we performed in vitro binding and gel mobility shift assays. Glutathione S-transferase (GST)-tagged Puf proteins were purified from *Escherichia coli* and incubated with radiolabeled RNA encompassing the *YHB1* 3' UTR PRE region (33 nt) with either the wild-type, site #1 mutant or site #2 mutant sequence (Figure 3.2A). The resulting complexes were separated on a native polyacrylamide gel. As shown in Figure 3.2C and graphically in Figure 3.2D, Pufp+RNA complex formation was reduced with the site #1 mutant RNA relative to wild-type, and even further reduced with the site #2 mutant RNA for all three Puf proteins, with Puf5p binding being most dramatically reduced. Thus, decreased decay stimulation of the mutant sites correlates with decreased binding capacity of the Puf proteins. To compare binding between the Puf proteins, increasing concentrations of Puf proteins were incubated with either the wild-type or site #1 radiolabeled RNA sequence, and complexes were analyzed on native polyacrylamide gels. As shown in Figure 3.2E, relative levels of Puf5p binding with wild-type RNA was significantly larger than with Puf1p or Puf4p, with Puf1p showing the least levels of binding. This pattern of differential binding was also seen with the site #1 RNA, but the relative differences between the Pufs were not as large. These experiments support the idea that differential binding capacity between Puf1p, Puf4p and Puf5p likely contributes to their differential roles in decay stimulation, especially for the lesser

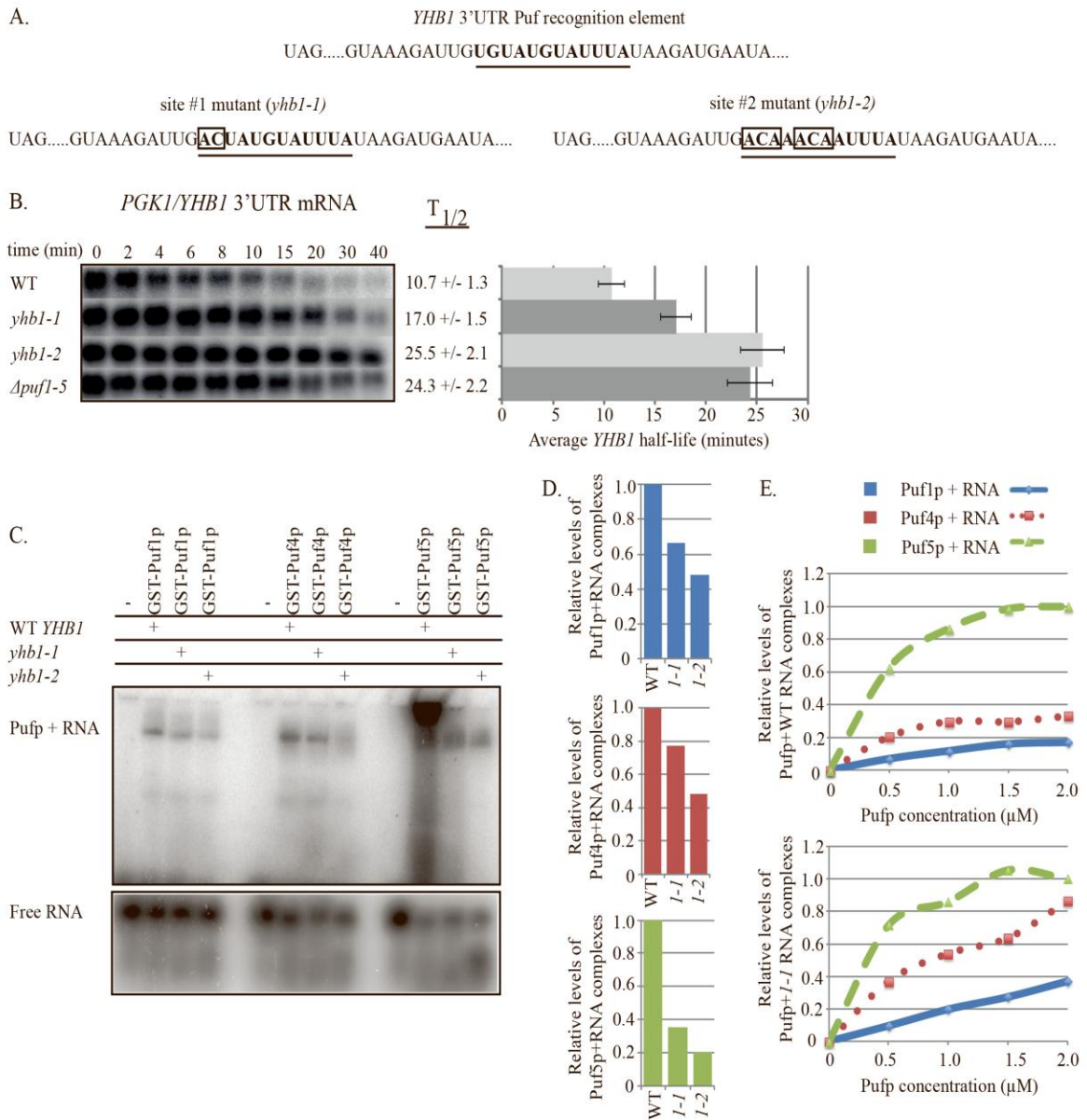


Figure 2.3. A single, flexible Puf recognition element in the *YHB1* 3' UTR is required for Puf1p, Puf4p and Puf5p regulated decay and binding. (A) Sequence of the Puf recognition element (bolded and underlined) in wild-type *YHB1* 3'UTR. Mutation of the first UGU to ACU, site #1 mutant, (*yhb1-1*) is represented to the left; mutation of both UGU elements to ACA, site #2 mutant, (*yhb1-2*) is represented to the right. Mutated sequences are boxed. (B) Decay of WT, *yhb1-1* mutant and *yhb1-2* mutant *PGK1/YHB1* 3' UTR fusion mRNA in WT yeast, or WT *PGK1/YHB1* 3' UTR in Δ *puf1-5* yeast. Representative Northern blots are presented in the left panel, with average half-life ($T_{1/2}$) listed to the right of each blot. Graphical representation of average half-life is presented in the right panel. Minutes following transcriptional shut-off are indicated above blots and along the X-axis of the graph. The error for each $T_{1/2}$ is the SEM ($n \geq 3$). (C) Representative gel mobility shift assay of the WT *YHB1*, *yhb1-1* mutant or *yhb1-2* mutant RNA sequence shown in (A) in the presence or absence of 1.5 mM GST-Puf1p, GST-Puf4p or GST-Puf5p. Positions of free radiolabeled RNA and RNA bound to a Puf protein (Pufp + RNA) are indicated. (D) Graphical representation of the data from (C), with relative levels of Pufp+RNA complexes for each Puf protein on the y-axes. Data is an average of two experiments. (E) Graphical representation of gel mobility shift assays in which increasing concentrations of Puf proteins were incubated with either WT RNA (top) or *yhb1-1* mutant RNA (bottom). Relative levels of Pufp+RNA complexes are indicated on the y-axis with Puf protein concentration on the x-axis. Data is an average of two experiments.

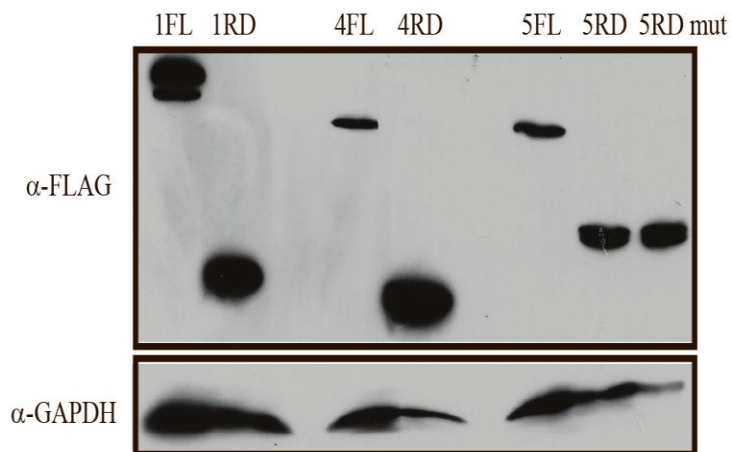


Figure 3.3. Expression of FLAG-tagged Puf proteins. Western blot analysis was performed on total cell extracts from yeast strains expressing either the full-length (FL) or repeat domain (RD) of FLAG-tagged Puf proteins 1,4 and 5. 5RD mut is the phosphomimetic mutant of Puf5p at residue 177. Anti-FLAG was used in the top panel to detect Puf overexpression levels, while anti-GAPDH was used in the lower panel as a loading control. Equal amounts of protein were added per lane based on a Bradford assay.

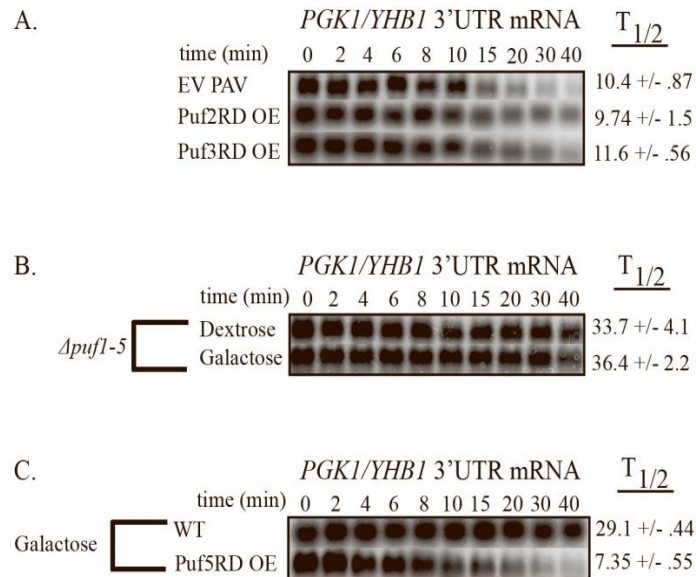


Figure 4.3. Decay analysis of the *PGK1/YHB1* 3' UTR reporter RNA. (A) Decay of WT *PGK1/YHB1* 3' UTR fusion mRNA in WT yeast co-expressed with empty vector (EV PAV), Puf2RD or Puf3RD. Overexpression (OE) constructs used are indicated to the left of the blots. (B) Decay of WT *PGK1/YHB1* fusion mRNA in a $\Delta puf1-5$ strain grown in dextrose or galactose (indicated to the left of the blot). (C) Decay of WT *PGK1/YHB1* fusion mRNA in the presence of galactose with no overexpression (WT) or Puf5RD OE. For A, B and C, representative northern blots are shown, with average half-life ($T_{1/2}$) listed to the right of each blot. Minutes following transcriptional shut-off are indicated above blots. For A, B and C the error for each $T_{1/2}$ is the SEM ($n \geq 3$).

role of Puf1p, though Puf4p appears to have a greater role in decay than its binding capacity would suggest.

Overexpression of Puf4p or Puf5p enhances decay of *YHB1* mRNA

To determine if overexpression of Puf proteins can increase the decay rate of *PGK1/YHB1* 3'UTR mRNA, we co-expressed our reporter construct with Puf overexpression constructs consisting of either the repeat domain (RD) or full length (FL) Puf protein in the WT strain. All FLAG-tagged constructs were validated for expression by western blot analysis (Figure 3.3). As expected from the deletion analysis, overexpression of Puf2p or Puf3p had no effect on the decay of our reporter (Figure 3.4). Unexpectedly, overexpression of either Puf1RD or Puf1FL had no effect on decay (Figure 3.5A), suggesting that Puf1p cannot enhance the normal decay stimulation by Pufs on this mRNA, likely due to its inferior binding. In contrast, overexpression of Puf4FL, Puf4FL/CEN vector, Puf5FL, or Puf5RD enhanced decay of the reporter, with Puf4FL/CEN having the greatest effect by decreasing the half-life >2 fold (Figure 3.5A). These data indicate that the levels of Puf4p and Puf5p are normally limiting the decay rate of *YHB1* mRNA, and/or these Pufs can out-compete wild-type levels of Puf1p for binding/decay stimulation. It is unclear why the Puf4RD did not enhance decay. It is possible that sequences outside the repeat domain are important for activity. Alternatively, all over-expression constructs were made in a 2 μ vector except the Puf4FL/CEN made in a CEN vector, and this Puf4FL/CEN showed greater activity in enhancing decay than Puf4FL from the 2 μ vector. While both the CEN and 2 μ vectors utilize the constitutive GPD promoter to express Puf4FL, it is possible that expression

from the high-copy 2 μ vector adversely affects Puf activity, perhaps through aggregation of the larger pool of Puf proteins. In contrast, the low-copy CEN vector may overexpress enough Puf4FL to enhance decay, but not so much as to aggregate. Moreover, Puf4RD is expressed \sim 7-fold higher than Puf4FL from the 2 μ vector (and \sim 3-fold higher than Puf5RD), which may contribute to its further aggregation and loss of activity (Figure 3.3). During the construction of the Puf5RD overexpression vector, a mutant was randomly generated during PCR at residue 177 of Puf5p, with asparagine changed to aspartic acid. This residue is directly downstream of a known phosphorylated residue at 176 (Bodenmiller *et al.*, 2010) (PhosphoPep). Overexpression of this Puf5RD mutant decreased the half-life of our reporter >2 fold, showing stronger activity than the wild-type Puf5RD protein (Figure 3.5A). Since expression of this mutant was not significantly different from the wild-type Puf5RD (Figure 3.3), this result suggests that the local charge and perhaps phosphorylation in this region enhances Puf5 activity.

Overexpression of Puf4p requires a functional PRE to enhance decay

We next sought to determine if the enhanced decay resulting from the overexpression of Puf proteins specifically acts through a functional PRE. Using our mutant reporter constructs, we performed transcriptional repression assays in the presence of Puf1FL, Puf4FL/CEN or Puf5FL overexpression and evaluated reporter half-life. With the site #1 mutant (*yhb1-1*), which normally decays with a half-life of 17.0 \pm 1.5 min (Figure 3.2B), both Puf4FL/CEN and Puf5FL could stimulate rapid decay to the same extent as with the wild-type *YHB1* 3'UTR (Figure 3.5B). These results indicate that Puf4p or Puf5p overexpression facilitates enhanced decay by using the second UGU element. Puf1FL

overexpression also rescued decay of the *yhb1-1* mRNA to near WT *YHB1* levels. Thus, while Puf1FL overexpression cannot enhance decay beyond the endogenous rate, it can facilitate decay of the mutant through the second UGU site. In contrast, when both UGU sites are mutated (*yhb1-2*), Puf4FL/CEN overexpression could not fully rescue decay, as the mutant *yhb1-2* showed a nearly 3-fold increase in half-life compared to WT (Figure 3.5B). These data suggest that the enhanced decay mediated by Puf overexpression depends on binding to a functional PRE. We also examined *HXK1* mRNA, a second known target destabilized by Puf1p, Puf4p and Puf5p (Ulbricht and Olivas, 2008). In the presence of Puf4FL/CEN overexpression, the half-life of the *PGK1/HXK1* 3'UTR construct was decreased 2-fold, indicating that the enhanced decay facilitated by overexpression of Puf4p is not limited to a single target mRNA (Figure 3.5C). While Puf4FL/CEN overexpression was unable to enhance decay of the *yhb1-2* mutant to the level of the WT construct, the decay of the *yhb1-2* mutant was still faster in the presence of Puf4FL/CEN overexpression as compared to endogenous levels of Puf proteins. It is possible that increased levels of Puf4p may allow for utilization of other UGU elements in the *YHB1* 3'UTR that are not consensus PREs (Figure 3.1A). To examine off-target effects of Puf4FL/CEN overexpression, we examined the decay rate of a *PGK1/PGK1* 3'UTR construct that is not under Puf protein regulation and contains no 3' UTR UGU elements (Ulbricht and Olivas, 2008). Puf4FL/CEN overexpression did not substantially decrease the half-life of this non-specific target, indicating that enhanced decay by Puf overexpression is specific to target mRNAs (Figure 3.5D).

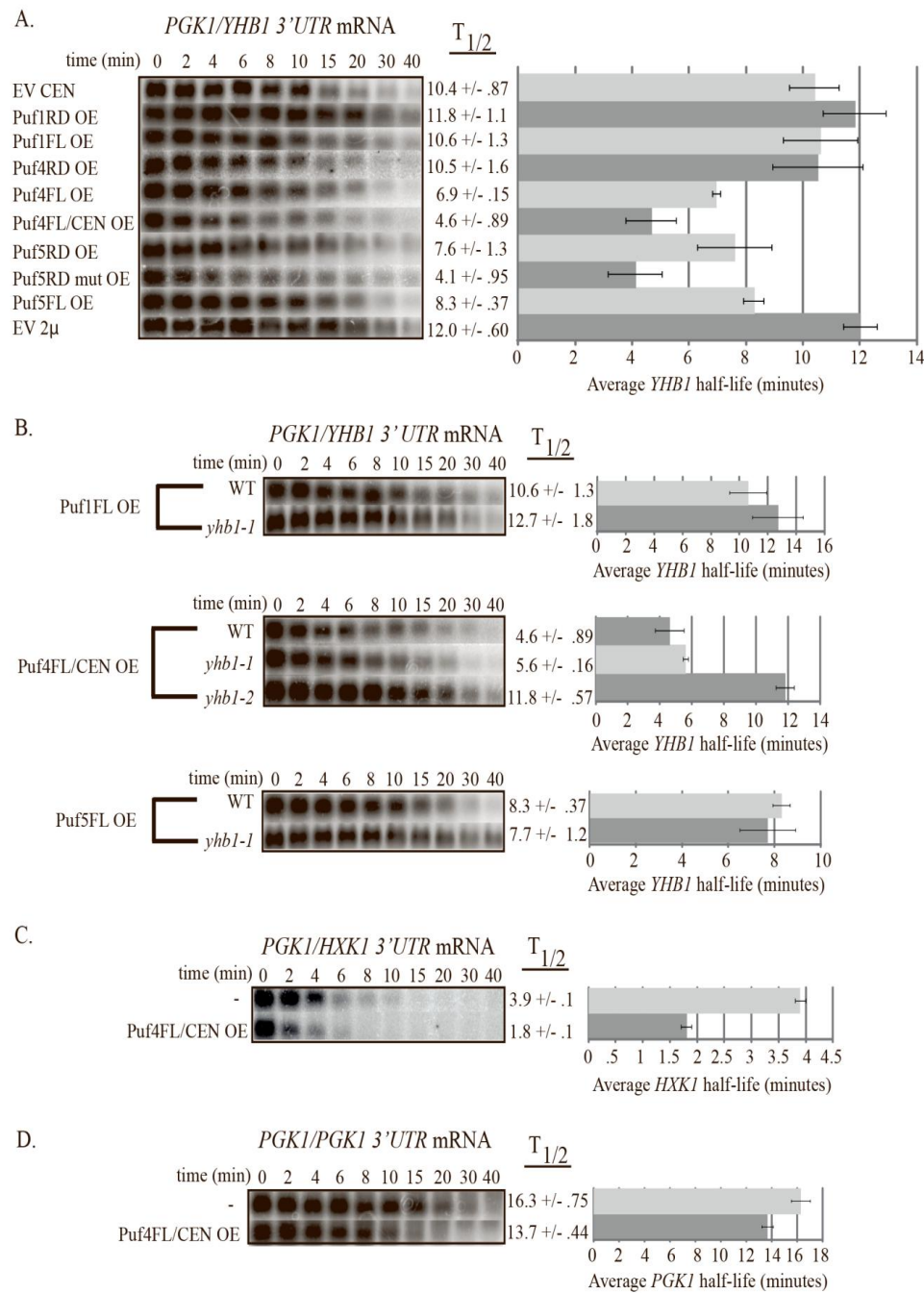


Figure 5.3. Overexpression of Puf4p or Puf5p in WT yeast stimulates a more rapid decay of target mRNAs. (A) Decay of WT *PGK1/YHB1* 3' UTR fusion mRNA in WT yeast co-expressed with empty vector (EV CEN or EV 2 μ), Puf1 full length (FL), Puf1 repeat domain (RD), Puf4FL, Puf4RD, Puf4FL/CEN, Puf5FL, Puf5RD or Puf5RD mutant (mut). Representative northern blots are shown in the left panel, with average half-life ($T_{1/2}$) listed to the right of each blot. Graphical representation of average half-life is presented in the right panel. Minutes following transcriptional shut-off are indicated above blots and along the x-axis of the graph. Overexpression (OE) constructs used are indicated to the left of the blots. (B) Decay of WT *PGK1/YHB1*, site #1 mutant (*yhb1-1*) or site #2 mutant (*yhb1-2*) in the presence of Puf1FL, Puf4FL/CEN or Puf5FL overexpression. (C) Decay of *PGK1/HXK1* 3' UTR fusion mRNA in the absence (-) or presence of Puf4FL/CEN. (D) Decay of *PGK1/PGK1* 3' UTR fusion mRNA in the absence (-) or presence of Puf4FL/CEN. For A, B and D the error for each $T_{1/2}$ is the SEM ($n \geq 3$). For C the error for each $T_{1/2}$ is the SEM ($n=2$).

Expression of Puf1p, Puf4p or Puf5p rescues decay of YHB1 mRNA in the absence of other Pufs

To evaluate the ability of individual Puf proteins to stimulate *YHB1* mRNA decay in the absence of other Puf proteins, we performed individual Puf rescue studies in the $\Delta puf1-5$ strain by co-expressing our reporter construct with constructs containing the full-length or repeat domain of Pufs 1, 4 or 5. In the absence of *PUFS* 1-5, exogenous overexpression of Puf1FL or its RD was able to rescue decay to levels similar to the 10.7 +/- 1.3 min half-life seen in the wild-type PUF strain (Figure 3.6A). Thus, while Puf1p overexpression was not able to enhance decay faster than wild-type rates when other Puf proteins are present, it is able to facilitate decay of *YHB1* on its own. Moreover, the repeat domain of Puf1p is sufficient for this activity. Exogenous expression of either Puf4FL or its RD, or Puf5FL or its RD was able to rescue decay to levels faster than that seen in the wild-type PUF strain (Figure 3.6A, B). These data illustrate that either the full-length or the repeat domain of Puf4p or Puf5p is individually sufficient to facilitate enhanced decay of *YHB1*, demonstrating no need for different Pufs to act synergistically when overexpressed, and further supporting the idea that endogenous levels of Puf proteins are limiting *YHB1* decay. Even alone, Puf4p and Puf5p are more active than Puf1p, likely due to their enhanced binding capacity. It is interesting that the Puf4RD facilitated enhanced decay in the absence of other Pufs, while it did not enhance the endogenous rate of decay in the presence of other Pufs. The Puf4RD that is available for binding (and not potentially aggregated) may be less able to compete with endogenous Puf4p and Puf5p for RNA binding.

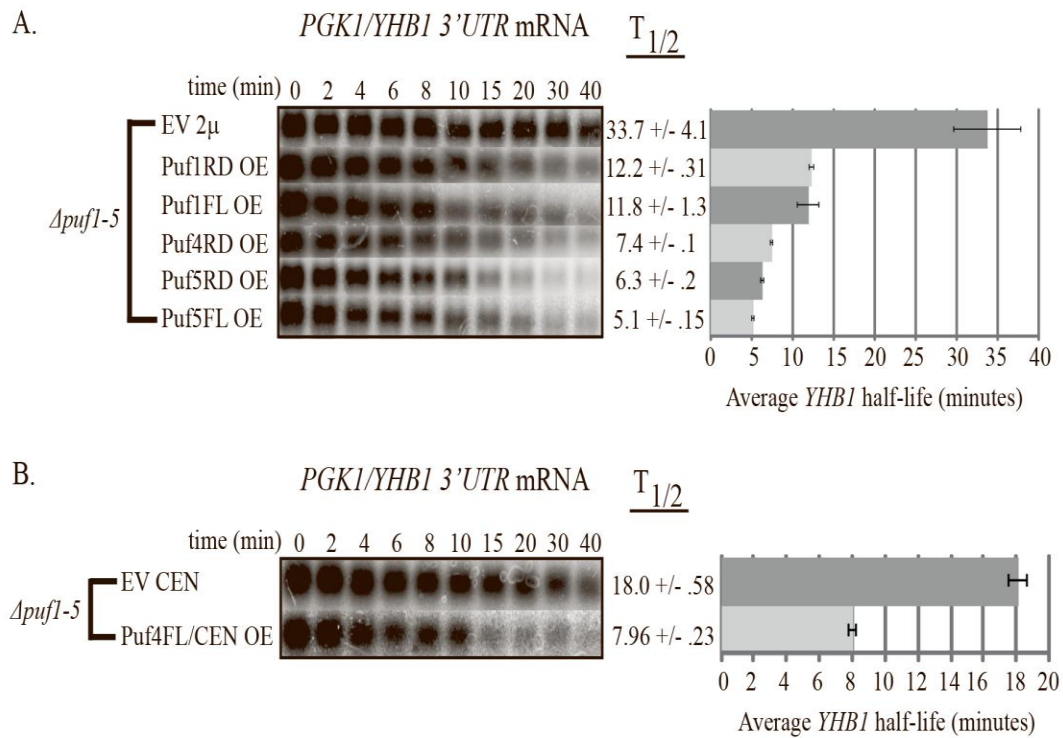


Figure 6.3. Expression of Puf1p, Puf4p or Puf5p or the corresponding repeat domain (RD) is sufficient to rescue decay of *YHB1* mRNA in a $\Delta puf1-5$ strain. (A) Decay of WT *PGK1/YHB1* fusion mRNA in a $\Delta puf1-5$ strain co-expressed with EV 2 μ , Puf1FL, Puf1RD, Puf4RD, Puf5FL or Puf5RD. Representative northern blots are shown in the left panel, with average half-life ($T_{1/2}$) listed to the right of each blot. Graphical representation of average half-life is presented in the right panel. Minutes following transcriptional shut-off are indicated above blots and along the x-axis of the graph. Overexpression (OE) constructs used are indicated to the left of the blots. (B) Decay of WT *PGK1/YHB1* fusion mRNA in a $\Delta puf1-5$ strain co-expressed with EV CEN or Puf4FL/CEN. For A the error for each $T_{1/2}$ is the SEM ($n \geq 3$) except for EV 2 μ where $T_{1/2}$ is the SEM ($n=2$). For B the error for Puf4FL/CEN $T_{1/2}$ is the SEM ($n \geq 3$) and the error for EV CEN $T_{1/2}$ is the SEM ($n=2$).

Stimulation of *YHB1* mRNA decay by Puf proteins is dependent on the available carbon source

Steady-state expression profiles of mRNAs containing putative PREs show altered expression levels in response to carbon source, suggesting that Puf protein regulation of these mRNAs is dependent on the available carbon source (Foat *et al.*, 2005). Yhb1p levels have been shown to decrease immediately upon glucose addition (Zhu *et al.*, 2006). This result is consistent with reduced mitochondrial respiration in the presence of glucose, which would decrease the production of NO and the need for Yhb1p. Post-transcriptional regulation of *YHB1* mRNA decay could contribute to such rapid changes in protein production. To assess the effects of carbon source on Puf-mediated decay of *YHB1* mRNA, we performed transcriptional repression assays of our reporter constructs using only temperature-shift to mediate repression in the continual presence of galactose, as compared to all of our prior analyses of *YHB1* decay in which dextrose was added at the time of transcriptional repression. In the presence of continual galactose, the *YHB1* half-life was extended 3-fold from that found in dextrose (Figure 3.7A), similar to the half-life of the $\Delta puf1-5$ strain. This result suggests that Puf-mediated decay is inhibited in galactose. To further examine the effect of carbon source on a target of Puf1p, Puf4p and Puf5p destabilization, we examined our *HXK1* reporter. Similar to *YHB1*, the *HXK1* reporter showed a 4-fold extension of half-life in the presence of galactose as compared to dextrose (Figure 3.7B). To verify that this extension is not a global effect on mRNA decay and confirm that transcriptional repression of the *GAL* UAS is complete with only temperature-shift, we evaluated the *MFA2/MFA2pG* 3'UTR reporter, which is not under the control of Puf proteins. This reporter decayed similarly

in both dextrose and galactose conditions, suggesting that changes in carbon source do not affect global mRNA decay (Figure 3.7C). This negative control also eliminates the possibility that the *GAL* UAS promoter is leaky when performing temperature-mediated transcriptional repression in the presence of galactose. Additionally, the *YHB1* half-life was similar in the $\Delta puf1-5$ strain in galactose or dextrose conditions, mirroring galactose conditions in a WT strain (Figure 3.4). These data suggest that decay stimulation by Puf1p, Puf4p and Puf5p is inhibited in respiration conditions (galactose) and active in fermentation conditions (dextrose). The results also imply that the switch from galactose to dextrose performed in all of our prior transcriptional repression assays leads to a very rapid activation of Puf-mediated decay. Given the rapid activation of Puf activity upon dextrose addition at the time of transcriptional repression, we hypothesize that a post-translational mechanism such as phosphorylation is responsible for changes in Puf activity. Such mechanisms may affect protein localization or mRNA binding affinity.

Stimulation of *YHB1* mRNA decay by Puf proteins is dependent on the culture density

Global expression studies suggest that culture density may also alter Puf regulation (Foat *et al.*, 2005). To test this possibility, transcriptional repression assays were performed at elevated OD₆₀₀ values as compared to an OD₆₀₀ of 1, which was used in our prior analyses. In cell density conditions of OD₆₀₀=2 and OD₆₀₀=3, we observed an extension of half-life ≥ 3 fold, similar to that observed in galactose conditions (Figure 3.7D). To control for any global effects of elevated OD₆₀₀ on mRNA decay, we examined the *MFA2/MFA2pG* reporter and observed identical half-lives at OD₆₀₀=1 and OD₆₀₀=2,

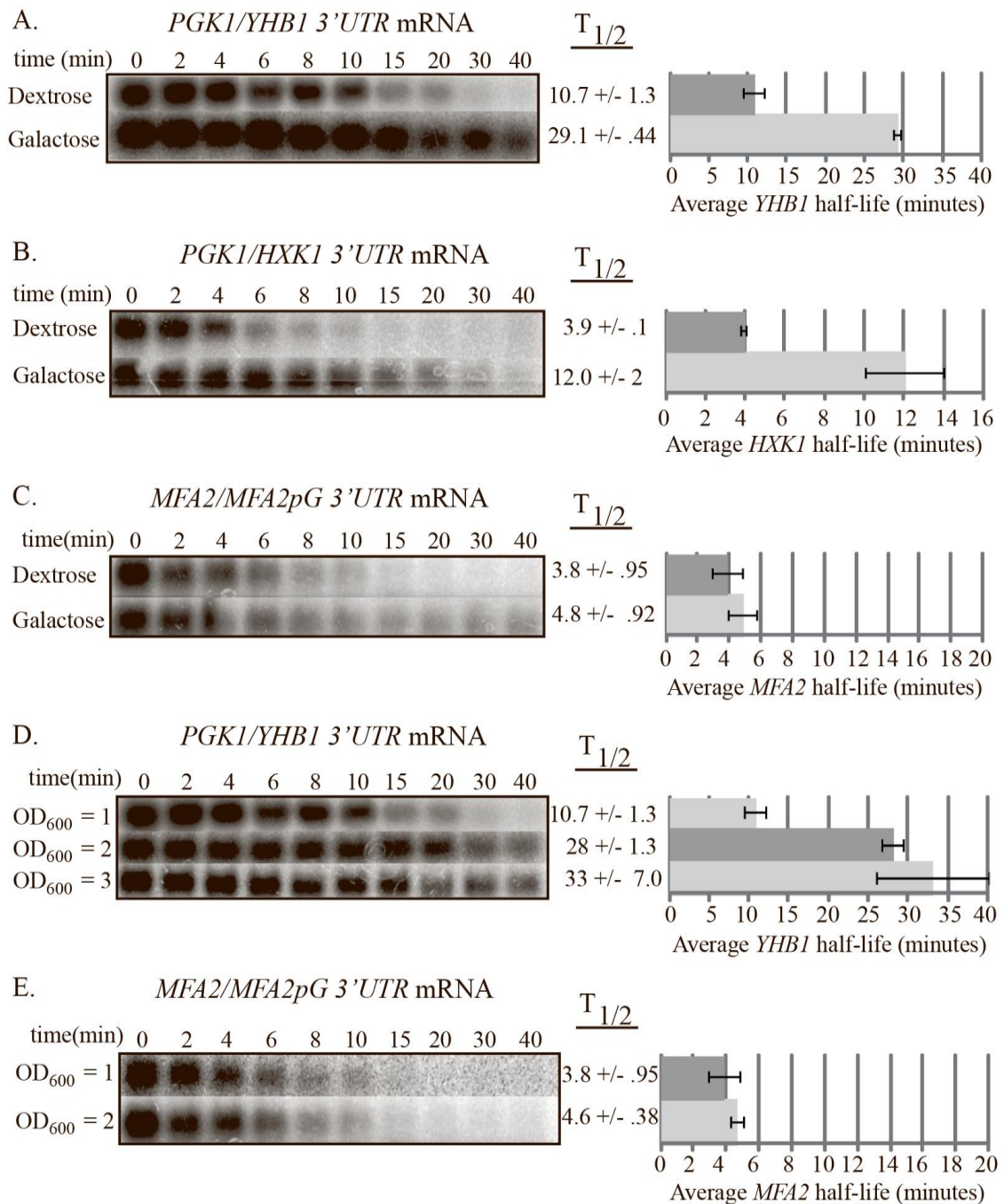


Figure 7.3. Regulation of *YHB1* mRNA stability by multiple Puf proteins is dependent on environmental conditions. Decay of *PGK1/YHB1* 3' UTR fusion mRNA (A), *PGK1/HXK1* 3' UTR fusion mRNA (B), or *MFA2/MFA2pG* 3' UTR fusion mRNA (C) in cultures grown in dextrose or galactose carbon sources. Decay of *PGK1/YHB1* 3' UTR fusion mRNA (D) or *MFA2/MFA2pG* 3' UTR fusion mRNA (E) grown to different final culture densities. Representative northern blots are shown in the left panels, with average $T_{1/2}$ listed to the right of each blot. Graphical representation of average half-life is presented in the right panels. Minutes following transcriptional shut-off are indicated above blots and along the x-axis of the graphs. For A-E the error for each $T_{1/2}$ is the SEM ($n \geq 3$).

indicating that the increase in mRNA stability is specific to targets under the control of Puf proteins (Figure 3.7E).

Overexpression of Puf proteins abrogates condition-specific inhibition of mRNA decay

To investigate whether overexpression of Puf protein levels can rescue Puf-mediated decay of target mRNAs in normally inactivating conditions, we first performed transcriptional repression assays in continual galactose in the presence of Puf4FL/CEN overexpression. The *YHB1* reporter decayed rapidly, resulting in a half-life 6-fold shorter than without Puf4FL/CEN overexpression (Figure 3.8A). Similar results were observed for Puf5RD overexpression (Figure 3.4). To validate this phenomenon with other Puf targets, we analyzed decay of our *HXK1* reporter. Again, rapid decay was rescued in continual galactose when Puf4FL/CEN was overexpressed (Figure 3.8B). We next examined if the inhibition of Puf-mediated decay at elevated culture density could be overcome by Puf overexpression. At the elevated OD₆₀₀=2, Puf4FL/CEN overexpression resulted in a 5-fold shorter half-life than without overexpression. These results suggest that the condition-specific Puf inactivation signal is limiting in cells (Figure 3.8C). The regulation of *YHB1* expression occurs at both transcriptional and post-transcriptional levels, and by multiple environmental signals. Transcriptional upregulation occurs in response to both NO exposure and the available carbon source through independent transcription factor mechanisms (Zhu *et al.*, 2006). We have shown that Puf1p, Puf4p and Puf5p destabilize *YHB1* mRNA post-transcriptionally in a carbon source and culture density dependent manner. To evaluate biological relevance of Puf regulation, we examined the effects of altering *YHB1* mRNA decay on cell fitness when exposed to exogenous NO. Previous studies have shown that a *yhb1Δ* strain exhibits severe growth

defects when exposed to 3mM DETA NONOate (Liu *et al.*, 2000). We hypothesized that overexpression of Puf4p would lead to a decrease in the pool of Yhb1p needed to combat exogenous NO stress, resulting in a growth defect. We first recapitulated the severe growth defect observed by Liu *et al.* in a *yhb1Δ* strain when exposed to 3mM DETA NONOate (Figure 3.8D). As predicted, overexpression of Puf4FL/CEN resulted in a significant reduction in growth upon DETA NONOate exposure (Figure 3.8D). Analysis of steady-state levels of endogenous *YHB1* mRNA showed reduced levels with Puf4FL/CEN overexpression and increased levels in a *puf4Δ* or *Δpuf1-5* strain compared to wild-type (Figure 3.8D). Finally, we evaluated endogenous *YHB1* mRNA decay in response to Puf overexpression. In a wild-type strain with empty vector, the half-life of endogenous *YHB1* mRNA was >40 minutes (Figure 3.8E). In contrast, Puf4FL/CEN overexpression in the wild-type strain showed a half-life of 13.5 ± 2 minutes. Overexpression of Puf5RD in the *Δpuf1-5* strain similarly increased decay from a >40 minute half-life to $14.6 \pm .34$ minutes. To gain insight into our hyperactive Puf5RD mutant, we overexpressed it in the *Δpuf1-5* strain and observed a 3-fold decrease in half-life beyond that observed with the wild-type Puf5RD (Figure 3.8E). These data demonstrate that altered mRNA levels resulting from changes to *YHB1* decay by Puf proteins influences the cell's response to toxic NO.

Puf protein inactivating conditions alters Puf5p mRNA binding.

To begin to dissect the mechanism by which Puf1p, Puf4p and Puf5p activity is altered by conditions, we first examined whether the expression levels of these Puf proteins are altered in different carbon sources. We utilized endogenously TAP-tagged

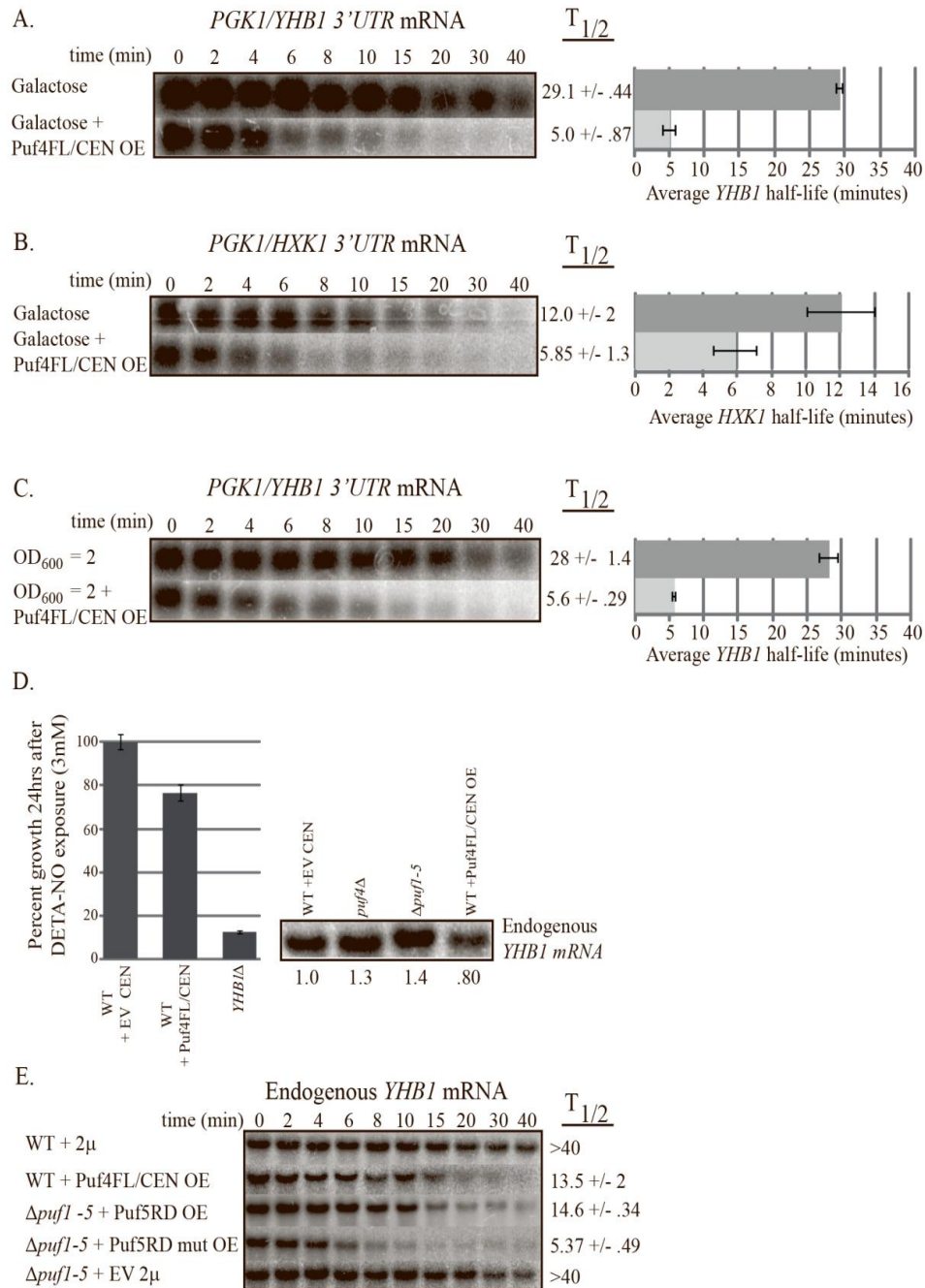


Figure 8.3. Overexpression of Puf proteins bypasses conditional regulation of *YHB1* mRNA stability. Decay of *PGK1/YHB1* 3' UTR fusion mRNA (A) or *PGK1/HXK1* 3' UTR fusion mRNA (B) in galactose in the absence or presence of Puf4FL/CEN overexpression. Representative northern blots are shown in the left panels, with average $T_{1/2}$ listed to the right of each blot. Graphical representation of average half-life is presented in the right panels. Minutes following transcriptional shut-off are indicated above blots and along the x-axis of the graph. (C) Decay of *PGK1/YHB1* 3' UTR fusion mRNA at OD₆₀₀=2 in the absence or presence of Puf4FL/CEN overexpression. (D) Percent cell growth of WT yeast expressing EV CEN or Puf4FL/CEN, or *yhb1Δ* yeast 24 hours after exposure to 3mM DETA-NO in galactose conditions is shown in the left graph. Percent growth is normalized to the growth of strains in the absence of DETA-NO. Northern blot analysis of endogenous, steady-state *YHB1* mRNA levels in WT yeast expressing EV CEN or Puf4FL/CEN, or *puf4Δ* yeast, or *Δpuf1-5* yeast is shown to the right. Levels of *YHB1* mRNA relative to the level in WT yeast expressing EV CEN and normalized for loading against scR1 RNA are shown below each lane. (E) Decay of endogenous *YHB1* mRNA. Representative northern blots are shown, with the average $T_{1/2}$ listed to the right of each blot. Yeast strain and expression vectors are indicated to the left of the blots. Minutes following transcriptional shut-off are indicated above the blots. For A-C the error for each $T_{1/2}$ is the SEM (n≥3). For D, the error is the SEM (n≥3). For E, the error for each $T_{1/2}$ is the SEM (n≥3).

Puf strains to evaluate steady-state Puf protein levels in cells grown in dextrose and galactose. Puf1p, Puf4p and Puf5p levels did not change between dextrose and galactose conditions, eliminating the possibility that decreased Puf activity in galactose is due to decreased protein levels (Figure 3.9A). This result supports our hypothesis that changes in Puf activity are due to a post-translational mechanism. Next, we investigated condition-specific localization of Puf1p-GFP, Puf4p-GFP and Puf5-GFP. No changes in localization of Puf1p, Puf4p or Puf5p were observed between dextrose and galactose conditions, eliminating altered localization as being responsible for Puf inactivation in galactose (Figure 3.9B). Finally, we assessed if mRNA binding was inhibited in galactose conditions. We used qPCR to quantitate the amount of mRNA that co-purified with TAP-tagged Puf1p, Puf4p and Puf5p from cells grown in dextrose or galactose. Both *YHB1* and *HXK1* mRNAs were analyzed as positive binding targets, while *CBS1* mRNA was analyzed as a negative control target to ensure we enriched for specific targets after Puf protein immunoprecipitation (IP). As shown in Figure 3.9C (top graphs), *YHB1* and *HXK1* were both enriched following IP with Puf1p, Puf4p and Puf5p from both dextrose and galactose conditions as compared to –RT control reactions (albeit to different levels), while there was no enrichment of *CBS1* mRNA. We next directly compared levels of *YHB1* and *HXK1* mRNAs that copurified with Puf proteins from dextrose or galactose conditions after normalizing for differences in IP efficiency of the Puf proteins and setting the levels found in galactose arbitrarily to 1 (Figures 3.9C bottom graphs, 9.3D). For both Puf1p and Puf4p, no significant changes in binding to either target mRNA could be detected between conditions, especially given the large difference in values obtained

from the two trials with *YHB1* mRNA as shown by the large error bars. In contrast, Puf5p binding to each target mRNA was consistently decreased in galactose conditions. Thus, condition-dependent binding to target mRNAs by Puf5p may contribute to its altered decay activity.

DISCUSSION

The flexibility of Puf proteins to regulate target mRNAs is increasingly evident. Systematic analyses identified 90 yeast transcripts that co-purified with more than one Puf protein (Gerber *et al.*, 2004). Several established targets of multiple Puf regulation contain multiple, nonoverlapping PREs, allowing simultaneous Puf binding (Hook *et al.*, 2007; Ulbricht and Olivas, 2008). Some of these PREs are also flexible enough to bind two different Puf proteins, including Puf1p and Puf5p alternatively binding a single site in *TIF1* mRNA (Ulbricht and Olivas, 2008), and Puf4p and Puf5p alternatively binding a single site in *HO* mRNA (Hook *et al.*, 2007). Since different Puf proteins favor binding to distinct PREs, the basis for this flexibility derives from the ability of Puf proteins to flip out one or more bases from the binding surface to accommodate non-canonical/extra bases within the 8 nucleotide binding site (Gupta *et al.*, 2008; Miller *et al.*, 2008; Koh *et al.*, 2009; Lu *et al.*, 2009; Valley *et al.*, 2012). This work investigates a novel target of multiple Puf regulation, *YHB1* mRNA, containing two overlapping PREs in its 3' UTR such that only one Puf protein can bind at one time. The first PRE in *YHB1* does not conform to either a Puf4p or Puf5p binding site, while the second PRE conforms to the canonical binding site for Puf5p (UGUANNNNUA) (Gerber *et al.*, 2004). Yet, *YHB1* is destabilized by Puf1p, Puf4p and Puf5p (Figure 3.1). Specifically, all three Pufs can bind and act

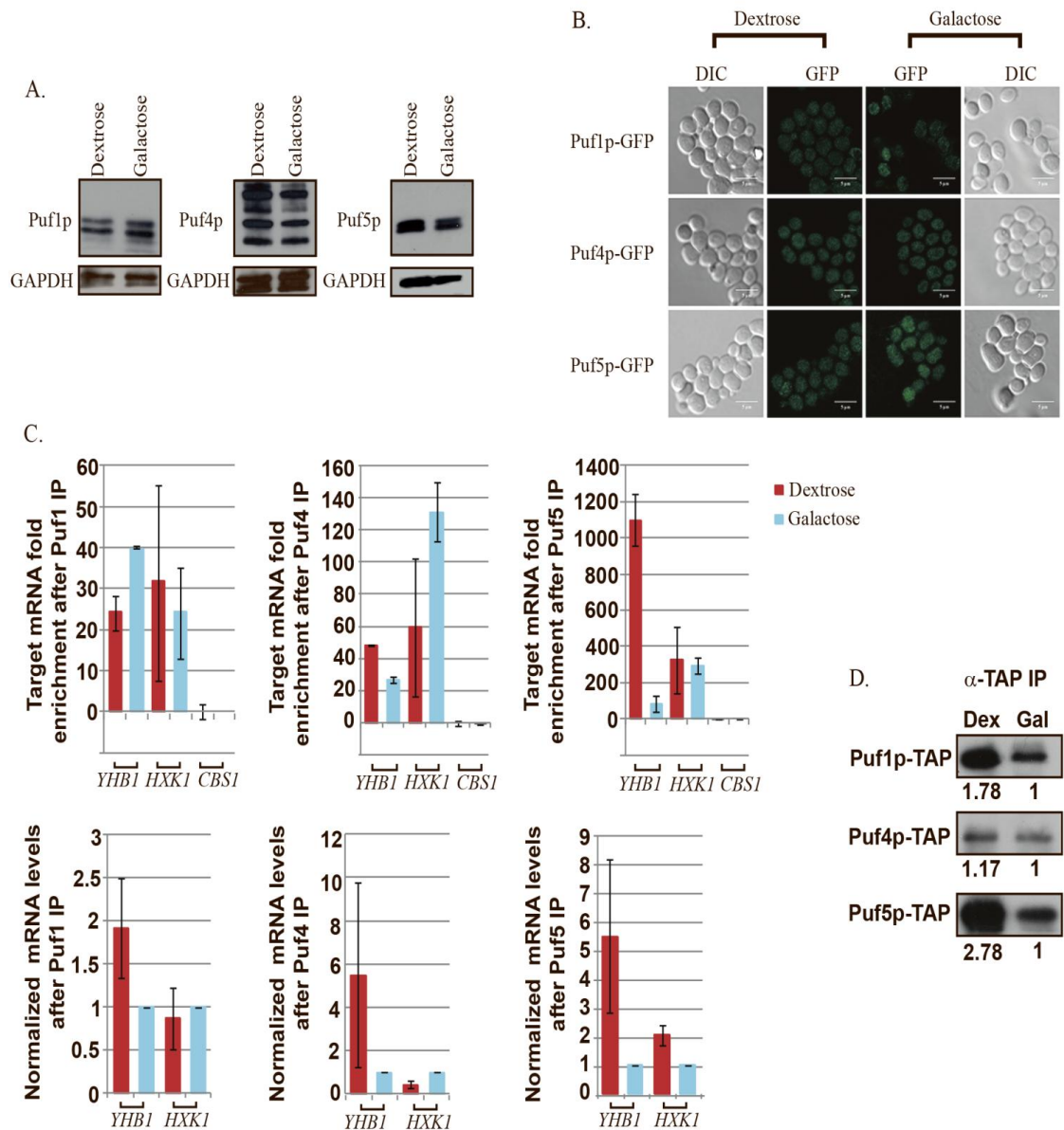


Figure 9.3. Effects of carbon source on Puf protein expression level, localization and mRNA binding. (A) Representative Western blot of Puf1p-TAP, Puf4p-TAP, or Puf5p-TAP levels in cultures grown in YEP media with 2% dextrose or galactose. GAPDH was detected as a loading control for all analyses. (B) Yeast expressing endogenously GFP-tagged Puf1p, Puf4p and Puf5p were grown in YEP media with 2% dextrose or galactose. Each image represents 10 flattened Z plane slices through fixed cells using confocal microscopy. Puf1-GFP, Puf4-GFP and Puf5-GFP are shown in green. Differential image contrasts are shown for reference (DIC). The bar equals 5mm. (C) Endogenously TAP-tagged Puf1p, Puf4p and Puf5p were immunoprecipitated (IP) from yeast grown in YEP media with 2% dextrose (red bars) or galactose (blue bars). Cq values after IP were compared to no reverse transcriptase (-RT) Cq values for each mRNA to acquire fold enrichment (top graphs). Cq values after IP in dextrose were compared to Cq values after IP in galactose and normalized to protein levels after IP to acquire mRNA levels bound after IP (bottom graphs). (D) Representative Western blot of protein levels after conditional IPs. Numbers below blots represent relative levels of protein after normalization. For (A) and (B) experiments were done in triplicate. For (C) and (D) experiments were done in duplicate.

through the second PRE, though both PREs contribute to full Puf-mediated decay stimulation (Figure 3.2, 3.5). It is proposed that Puf4p and Puf5p both bind to the sequence (UGUANNAUA), where the N bases at nucleotides 5 and 6 flip out from the Puf binding surface, while only Puf4p binds the sequence (UGUANANUA), where nucleotides 5 and 7 flip out (Valley *et al.*, 2012). However, neither overlapping PRE in *YHB1* conforms to either of these sequences, illustrating the uniqueness of *YHB1* as a target of multiple Puf regulation and implicating additional modes of base recognition and base flipping by Puf1p, Puf4p and Puf5p than previously described.

The binding and destabilization of *YHB1* mRNA by Puf1p, Puf4p and Puf5p indicate competition between Pufs. The extension of half-life in the *puf1Δ* strain was smaller than in the *puf4Δ* or *puf5Δ* strains, implying different binding and/or regulatory properties between Pufs. Our in vitro binding data suggest that inferior binding of Puf1p to the *YHB1* PREs likely contributes to its lesser role in decay stimulation, while the superior binding of Puf5p likely contributes to its larger role in decay. We also demonstrate that endogenous levels of Puf proteins are limiting, as overexpression of Puf4p or Puf5p in a wild-type strain facilitates more rapid decay of *YHB1* mRNA compared to wild-type (Figure 3.5A). In contrast, Puf1p overexpression in a wild-type strain had no effect on *YHB1* mRNA decay, which is likely a result of its inferior binding activity. However, any single deletion of *PUF1*, *PUF4*, or *PUF5* results in a decay phenotype, demonstrating that each Puf is important for fine-tuned regulation of *YHB1*.

Overexpression of a mutant form of the Puf5RD enhanced *YHB1* decay better than WT Puf5RD (Figure 3.5). This N→D mutation at residue 177 neighbors a known

phosphorylated serine at 176 (Bodenmiller *et al.*, 2010) (PhosphoPep). We hypothesize that the increased activity is due to the constitutive charge mimic in this region. Phosphorylation of Puf proteins has been shown to either stimulate activity, such as the case for human Pum1 (Kedde *et al.*, 2010), or inhibit activity as seen with yeast Puf6 (Deng *et al.*, 2008). Future research will evaluate the potential role of Puf5p phosphorylation in activity. Differential phosphorylation could lead to altered RNA binding or altered interactions with other proteins.

In the absence of other Pufs, overexpression of Puf1p, Puf4p or Puf5p is sufficient to rescue decay of *YHB1* mRNA (Figure 3.6). Thus, in the absence of competition from other Pufs, Puf1p is capable of binding and stimulating decay, even though it is not normally as active as Puf4p or Puf5p. The repeat domains of Puf1p, Puf4p and Puf5p are also sufficient to rescue decay of *YHB1* mRNA, demonstrating that all sequences necessary for both binding and decay stimulation are present (Figure 3.6).

Yhb1p is the only known defense against NO stress in *Saccharomyces cerevisiae* (Liu *et al.*, 2000). Upon exposure to NO, *YHB1* expression is increased due to transcriptional activation as well as post-transcriptional regulation (Foster *et al.*, 2009). Here we demonstrate that inhibition of Puf-mediated decay contributes to the stabilization and increased levels of *YHB1* mRNA in respiratory conditions (Figure 3.7). During respiration, mitochondrial cytochrome oxidase produces NO proportional to the cellular respiration level (Li *et al.*, 2011)). We have previously shown that decay stimulation by Puf3p, but not mRNA binding, is inhibited by respiratory conditions such as galactose (Miller *et al.*, 2014). This inactivation allows stabilization of Puf3p target

mRNAs encoding mitochondrial proteins, therefore increasing mitochondrial production under respiratory conditions (Figure 3.10). Like Puf3p, we propose that Puf1p, Puf4p and Puf5p are also inactivated in respiratory conditions. Such inactivation would allow for the increased production of Yhb1p to rapidly combat toxic NO levels accumulating in the cell (Figure 3.10). The stress of high cell density also inactivates Puf1p, Puf4p and Puf5p, resulting in stabilization of *YHB1* mRNA to combat this otherwise toxic event (Figure 3.7). In fermentation conditions or without exogenous stress, Puf1p, Puf3p, Puf4p and Puf5p are active to stimulate rapid turnover of their target mRNAs, whose protein products are not needed under these conditions.

We further demonstrate the biological relevance of this mechanism by increasing the decay rate of endogenous *YHB1* mRNA by Puf4p overexpression during exposure to exogenous NO, which resulted in decreased cell growth (Figure 3.8). To correlate this growth phenotype with levels of *YHB1* mRNA, we determined that steady-state levels of endogenous *YHB1* mRNA increase in *puf4Δ* and *Δpuf1-5* strains, but decrease with Puf4p overexpression (Figure 3.8). Overexpression of Puf4p could stimulate rapid decay of *YHB1* mRNA even in galactose and high cell density, suggesting that the high levels of Puf4p were overwhelming normal inactivation of Puf regulatory function (Figure 3.8). These results highlight the importance of the precise balance of Puf proteins normally in the cell to promote decay or respond to inactivation signals, possibly phosphorylation changes, under stress conditions.

Condition-specific inhibition of Puf protein activity could be due to decreased Puf expression, altered localization, reduced mRNA binding capacity, disruption of

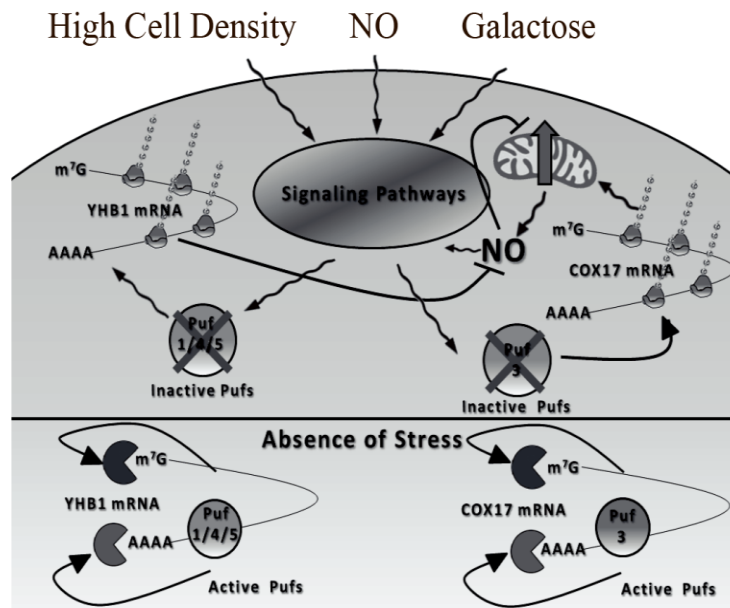


Figure 10.3. Model for Puf protein activity in the presence or absence of environmental stress. During exposure to environmental stresses including high cell density, nitric oxide (NO), and galactose, unknown signaling pathways inactivate Puf1p, Puf4p and Puf5p, resulting in up-regulation of *YHB1* mRNA levels and protein production, allowing for protection from toxic NO levels. Additionally, Puf3p activity is inhibited during exposure to galactose, resulting in increased mRNA stability of *COX17* mRNA and other mRNAs involved in mitochondrial development and function to accommodate the respiration demand. In the absence of stress, Puf proteins are active and repress target mRNAs.

interactions with other proteins, or altered activity of protein co-factors. We found that the inhibition of Puf1p, Puf4p and Puf5p activity in galactose is not due to decreased expression or altered localization. Examination of Pufp-mRNA binding interactions in vivo demonstrated that Puf1p and Puf4p bind target mRNAs in both activating and inactivating conditions. However, Puf5p showed reduced binding in inactivating conditions, which likely contributes to the condition-specific inhibition of Puf-mediated decay stimulation of *YHB1* given that Puf5p plays the largest role in *YHB1* decay. Such reduced binding by Puf5p is a unique mechanism of inactivation, as previous studies with Puf3p demonstrated that its mRNA binding was enhanced in galactose conditions (Miller *et al.*, 2014). Further research will dissect the mechanism of Puf5p binding inhibition, as well as possible changes to the interactions with or activity of protein co-factors.

In this work, we have elucidated a key role for Puf proteins in response to elevated levels of NO whereby stabilization of a specific target mRNA (*YHB1*) leads to increased cell fitness. Puf proteins may also play a role in translational inhibition of *YHB1* mRNA. Previous work has demonstrated that Puf4p acts exclusively through stimulation of deadenylation, while Puf5p can repress mRNAs through additional mechanisms, including translational inhibition (Hook *et al.*, 2007; Chritton and Wickens, 2010). A global study to identify mRNAs whose translation is dependent on Eap1p identified *YHB1*, and Puf activity has been shown to be dependent on Eap1p (Cridge *et al.*, 2010; Blewett and Goldstrohm, 2012). It is plausible that Puf proteins also

translationally repress *YHB1* mRNA through Eap1p by disrupting interactions with the initiation machinery and promoting decapping.

MATERIALS AND METHODS

Oligonucleotides, Plasmids and Yeast Strains

All yeast strains, plasmids and oligonucleotides are listed in Supplementary Tables 1, 2, and 3, respectively. Strain yWO268 was made by transforming yWO204 with pWO183 after digestion with BamHI to replace *URA3* with *KanMX3*. Transformants were plated on YPD plates containing 300µg/mL geneticin. Colonies were isolated and back-plated on media lacking uracil to verify loss of *URA3*. Strain yWO269 was made by replacement of *YHB1* with *URA3* in yWO7. *URA3* was amplified from pWO15 with *YHB1* flanking regions using primers oWO77 and oWO78. The PCR product was transformed into yWO7, with selection on media lacking uracil. Deletion of *YHB1* was verified by colony PCR and northern blot analysis.

PUF Overexpression Plasmids

The *PUF1* ORF was amplified from genomic DNA using primers oWO466 and oWO467. The *PUF1RD* was amplified from pWO48 with primers oWO468 and oWO145. The PCR products were inserted into the BamHI and Sall sites of pWO15 3' of the FLAG tag and under the control of the constitutive *GPD* promoter to create pWO114 (*PUF1* full-length) and pWO115 (*PUF1RD*).

PUF2RD was amplified from genomic DNA using primers oWO570 and oWO571. The PCR product was inserted into the BamHI and Sall sites of pWO15 to create pWO192.

pWO16 was created by removing the *PUF3RD* from pWO14 using BamHI and Sall and inserting into the respective sites in pWO15.

The *PUF4* ORF was amplified from genomic DNA using primers oWO610 and oWO611. The *PUF4RD* was amplified from genomic DNA using primers oWO638 and oWO611. The PCR products were inserted into the BamHI and Sall sites of pWO15 to create pWO193 (*PUF4RD*) and pWO194 (*PUF4* full-length). pWO116 (pRS415-*GPD-PUF4*) was a kind gift from Marvin Wickens.

The *PUF5* ORF was amplified from pWO18 using primers oWO612 and oWO613. The *PUF5RD* was amplified from pWO18 using primers oWO568 and oWO569. The PCR products were inserted into the BamHI and Sall sites of pWO15 to create pWO195 (*PUF5RD*), pWO196 (*PUF5* full-length) and pWO200 (*PUF5RDmut*). All constructs were verified by sequencing.

Protein Expression and Purification

The GST-*PUF1* fusion construct (pWO201) was made by PCR amplification of Puf1 with BamHI and NotI restriction sites from yeast genomic DNA using primers oWO865 and oWO866. The resulting fragment was then inserted into pGEX-3X (Amersham Biosciences) and validated by sequencing and western blot. The GST-*PUF4* fusion construct (pWO202) was made similarly with the modification of using restriction sites EcoRI and NotI, and primers oWO867 and oWO868. The GST-PUF fusion constructs pWO18, pWO201, and pWO202 were transformed into the protease-deficient *E. coli* strain BL-21 and GST fusion proteins were purified as recommended by the

manufacturer. Protein eluates were dialyzed into 50mM Tris-HCL (pH 8.0), and expression products were verified by Western blot analysis with anti-GST antibody.

PGK1-YHB1 3' UTR Reporter Plasmids

pWO127 (*URA3* marker) and pWO128 (*LEU2* marker) were made by PCR amplification of the *YHB1* 3' UTR from genomic DNA using primers oWO262 and oWO263. For pWO127, the PCR product was inserted into pRS227 (Heaton *et al.*, 1992)3' of the *PGK1* ORF between the *SacI* and *HindIII* sites. For pWO128, the *PGK1-YHB1 3'UTR* insert was cut from pWO127 and inserted into pWO61 between the *PvuII* sites.

pWO129 (*URA3* marker) was created by PCR-based in vitro site-directed mutagenesis of the *YHB1* 3' UTR PRE sequence UGUAUGUA to ACAAACAA using primers oWO480 and oWO481, in accordance with manufacturers recommendations (Stratagene QuikChange XL site-directed mutagenesis kit). pWO197 (*URA3* marker) was created by site-directed mutagenesis of the PRE sequence UGUAUGUA to ACUAUGUA using primers oWO513 and oWO514. pWO199 (*LEU2* marker) was made by performing a *URA3* to *LEU2* marker swap on pWO197 using pWO162 (ATCC product #87552) according to ATCC instructions. All constructs were verified by sequencing.

In Vivo YHB1 Decay Analysis of Puf Deletion Mutants

Decay of reporter mRNAs was monitored in strains harboring the temperature-sensitive *rbp1-1* RNA polymerase II allele, in which transcription is rapidly halted following a shift from 24°C to 37°C. All yeast transformations were performed by LiOAc high-efficiency transformation (Gietz *et al.*, 1995).

Transcriptional shut-offs were performed as described (Caponigro *et al.*, 1993) in yeast strains containing pWO127 or pWO128, which express a fusion RNA containing the *PGK1*Δ82 ORF and *YHB1* 3'UTR, with transcription regulated by the GAL UAS. pWO127 was transformed into yWO7 (WT), yWO102 (*puf1*Δ), yWO43 (*puf3*Δ), yWO105 (*puf4*Δ) and yWO268 (Δ*puf1-5*). pWO128 was transformed into yWO48 (*puf2*Δ), yWO49 (*puf5*Δ) and yWO204 (Δ*puf1-5*). Transcriptional shut-offs of *PGK1/yhb1* mRNA mutants were performed in yWO7 (WT) containing pWO129 or pWO197. Strains were grown as 200 mL cultures in selective minimal media containing 2% galactose at 24°C to an OD₆₀₀ of 1.0. Half of each culture was harvested and resuspended in 20 mL selective media containing 8% dextrose at 37°C, effectively shutting off transcription by both the temperature-sensitive inactivation of RNA pol II as well as carbon source inactivation of the GAL UAS. Time course samples were taken over a 40 minute time period at 37°C. Total RNA was isolated from yeast samples as described (Caponigro *et al.*, 1993), followed by northern blot analysis (Biobond plus nylon membrane-Sigma). Northern blots were probed with ³²P-end-labeled oWO105, oWO159 or oWO447 to detect *HXK1* 3' UTR, *YHB1* 3'UTR, or *PGK1* 3' UTR, respectively. Loading was normalized using *scR1* RNA, a constitutively expressed RNA polymerase III transcript, and all imaging and quantification of half-lives were determined by ImageQuant software (Molecular Dynamics).

In Vitro Binding Assays

RNAs representing the wild-type *YHB1* 3'UTR, *yhb1-1* mutant 3'UTR and *yhb1-2* mutant 3'UTR were purchased from IDT (oWO 898, oWO899, and oWO900,

respectively). The RNAs were subjected to 32 P-end-labeling for detection in the binding assay. Binding reactions included radiolabeled RNA (100,000 counts per minute) and 1X binding buffer (10 mM HEPES pH 7.5, 50 mM KCl, 1 mM EDTA, 2 mM dithiothreitol (DTT), 200 U/ml RNasin ribonuclease inhibitor, 0.1 mg/ml bovine serum albumin, 0.01% Tween-20, 0.1 mg/ml poly(rU), and 10 μ g/ml yeast tRNA) in the presence or absence of GST-Puf1p, GST-Puf4p or GST-Puf5p in a total of 20 μ l. Reactions were incubated for 30 min at 24°C, 5 μ g of heparin was added and reactions incubated for a further 10 min at 24°C, then reactions were electrophoresed on 8% nondenaturing polyacrylamide gels for 2.5 h at 200 V at 4°C. To calculate relative binding, storage phosphor signals were first determined for each bound complex, and background signal from the no protein lane was subtracted from each. For comparison of binding between RNA targets, values for each set of Puf+RNA complexes were divided by the corresponding Puf+WT RNA value, then normalized values were averaged between trials. For comparison of binding between Puf proteins on a single RNA target, values for each set of Puf-RNA complexes were divided by the 2 μ M Puf5p+RNA value, then normalized values were averaged between trials.

In Vivo *YHB1* Decay Analysis with Puf Overexpression

Transcriptional shut-offs were performed as described above in yWO7 co-transformed with plasmids pWO100, pWO102, pWO127, pWO128, pWO129, pWO197 or pWO199 and Puf overexpression plasmids pWO15, pWO16, pWO58, pWO114, pWO115, pWO116, pWO192, pWO193, pWO194, pWO195, pWO196 or pWO200.

Transcriptional shut-offs were also performed in yeast strains yWO204 ($\Delta puf1-5$) and yWO268 ($\Delta puf1-5$) co-transformed with plasmids pWO127 or pWO128 and Puf overexpression plasmids pWO15, pWO16, pWO58, pWO114, pWO115, pWO116, pWO192, pWO193, pWO194, pWO195 or pWO196. Similar detection and quantification methods were used as described above.

In Vivo Decay Analysis in Alternate Carbon Sources or Culture Densities

Transcriptional shut-offs were performed in yWO7 (WT) transformed with pWO61, pWO100 or pWO127; or yWO204 ($\Delta puf1-5$) transformed with pWO128. To examine decay in cultures grown continuously in galactose, strains were grown as 200 mL cultures in selective minimal media containing 2% galactose at 24°C to an OD₆₀₀ of 1.0. Half of each culture was harvested and resuspended in 20 mL selective media containing 8% galactose at 37°C, effectively shutting off transcription by the temperature-sensitive inactivation of RNA pol II. To examine decay in cultures grown to higher cell densities, strains were grown as 200 mL cultures in selective minimal media containing 2% galactose at 24°C to an OD₆₀₀ of 2.0 or 3.0. 50 mL (OD₆₀₀ 2.0) or 25 mL (OD₆₀₀ 3.0) of cells were harvested and resuspended in 20 mL selective media containing 8% dextrose at 37°C, effectively shutting off transcription by both the temperature-sensitive inactivation of RNA pol II as well as carbon source inactivation of the GAL UAS. Similar detection and quantification methods were used as described above. Northern blots were probed with ³²P-end-labeled oWO105, oWO159 or oWO238 to detect *HXK1* 3' UTR, *YHB1* 3' UTR or *MFA2pG* 3' UTR, respectively.

In Vivo Endogenous *YHB1* mRNA Decay Analysis

Transcriptional shut-offs were performed in yWO7 or yWO268 transformed with over-expression plasmids pWO15, pWO58, pWO116, pWO195 or pWO200. Strains were grown as 200 mL cultures in selective minimal media containing 2% galactose at 24°C to an OD₆₀₀ of 1.0. Half of each culture was harvested and resuspended in 20 mL selective media containing 8% dextrose at 37°C, effectively shutting off transcription by the temperature-sensitive inactivation of RNA pol II. Similar detection and quantification methods were used as described above.

Growth inhibition study

Mid-log phase (OD₆₀₀ 0.4-0.6) cells were diluted to an OD₆₀₀ of 0.04 and allowed to grow at 24°C for 24hrs during exposure to 3mM DETA-NO. Cell growth was monitored every 4hrs by OD₆₀₀ measurements. Growth percentage represents growth with 3mM DETA-NO exposure normalized against growth with no added DETA-NO for each individual strain (yWO7 transformed with pWO58 or pWO116, or yWO269).

Steady-state Detection of Endogenous *YHB1* mRNA

Mid-log phase (OD₆₀₀ 0.4-0.6) cells were harvested and total RNA was extracted from yWO7 transformed with pWO58 or pWO116, yWO105, or yWO204. Northern analysis and detection of *YHB1* mRNA with oWO159 was performed. Similar detection and quantification methods were used as described above.

Puf1p, Puf4p and Puf5p Western Analysis

Protein extracts were prepared from 20 ml yeast cultures of endogenously TAP-tagged strains TAP-Puf1p, TAP-Puf4p and TAP-Puf5p (Ghaemmaghami *et al.*, 2003)

(Thermo-Fischer Scientific) grown in YEP containing 2% dextrose or galactose at 30°C to an OD₆₀₀ of 1.0. Harvested cells were resuspended in 0.25 ml of sample buffer [125 mM Tris-HCl, pH 6.8, 1% sodium dodecyl sulphate (SDS), 2% glycerol, 10% beta-mercaptoethanol (BME)], lysed with glass beads, and extract collected by poking a hole in the bottom of the microfuge tube and spinning into a 15-ml centrifuge tube. Equal OD₆₀₀ units of total protein were loaded onto a 10% Tris-glycine polyacrylamide gel (Bio-Rad). Resulting gels were blotted to nitrocellulose and probed with anti-TAP antibodies. Blots were also probed with anti-GAPDH (Thermo Scientific) antibodies as a loading control.

Confocal Fluorescent Microscopy

Endogenously green fluorescent protein (GFP)-tagged Puf1p, Puf4p and Puf5p (yWO200, yWO203, yWO199) (Huh *et al.*, 2003) were grown at 30°C to an OD₆₀₀ of 1.0 in YEP supplemented with 2% dextrose or galactose. Cells were then fixed with 3.7% formaldehyde for 1 hr. The cells were washed twice with 1x phosphate buffered saline (PBS) and resuspended in 1 ml of 1x PBS. 10 milliliters of cells was loaded onto a circular glass slide coated with 1% polyethyleneimine (SigmaAldrich Cat# P3143-100ML) and cells were allowed to settle for 10 min. The cell solution was aspirated from the edge of the slide, and the slide was then dipped in 1x PBS twice to remove nonadherent cells. A coverslip was applied and the cells were immediately visualized with a Zeiss LSM-700 confocal microscope on the 100x oil immersion objective for eGFP fluorescence. Ten slices were taken through the Z plane of the cells. The slices were flattened using Fiji Is Just ImageJ (FIJI) Z project at maximum intensity, and the intensity of GFP signal was

adjusted such that the maximum signal for cells grown in galactose was set as the maximum for cells grown in dextrose.

Quantitative real-time PCR of RNA associated with Puf1p, Puf4p and Puf5p

RNA immunoprecipitation (IP) was performed essentially as described (Gerber *et al.*, 2004), with minor alterations. Endogenously TAP-tagged Puf1p, Puf4p and Puf5p cells (yWO272, yWO271, yWO270) were grown at 30°C in 1L YEP supplemented with 2% dextrose or galactose to an OD600 of 1.0. The cells were pelleted, washed twice with 25 ml of ice-cold Buffer A (20 mM Tris-HCl pH 8.0, 140 mM KCl, 1.8 mM MgCl₂, 0.1% Nonidet P-40, 0.02 mg/ml heparin) and frozen at -80°C. The following day, cells were thawed on ice and resuspended in 5 ml of Buffer B (Buffer A with 0.5 mM DTT, 1× Complete Mini Protease Inhibitors [Roche Diagnostics Ref#11-836-153-001], 40 U/ml RNasin ribonuclease inhibitor, 0.2 mg/ml heparin). Cells were vortexed in the presence of glass beads for 1 min and placed on ice for 1 min for a total of five times. Lysates were clarified by centrifuging at 7000g for 5 min. The supernatant was collected and protein concentration was measured using a standard Bradford assay. Lysates were normalized to contain 1.625 mg in a volume of 5 ml, and bovine serum albumin (BSA) to 1% and 50 µg yeast tRNA were added. The lysates were incubated with 400 µl of 50% IgG Sepharose 6 Fast Flow (GE Healthcare Cat# 17-0969-010) that had been blocked for 1 h in 1 ml of Buffer B plus 1% BSA and 50 µg of yeast tRNA. Beads were incubated at 4°C for 2 h, washed once with 5 ml of Buffer B for 15 min and three times with 5 ml of Buffer C (20 mM Tris-HCl pH 8.0, 140 mM KCl, 1.8 mM MgCl₂, 0.01% Nonidet P-40, 0.5 mM DTT, 12 U/ml RNasin Ribonuclease Inhibitor) for 15 min each. Beads were

resuspended in 400 μ l of Buffer C and 80 U ProTEV Plus (Promega Cat#V6101) was added. Beads were incubated for 2 h at 16°C. The resultant supernatant was drawn off and total RNA was isolated via hot phenol extraction. Glycogen (20 μ g) was added to the final aqueous phase to assist precipitation of RNA before the addition of one-tenth volume 3M NaOAc and 2.5 volumes ethanol. The entirety of immunoprecipitated RNA was subjected to DNase treatment via manufacturer's instructions (Ambion Turbo DNase). Reverse transcription of RNA was performed according to manufacturer's specifications (Biorad iScript). Quantitative PCR (qPCR) was optimized and performed on a Biorad CFX96 Real-Time system using SYBR Green detection chemistry (Biorad SSO advanced). Gene-specific qPCR primers are provided in Supplementary Table S3. All RT-qPCR experiments were conducted in technical triplicates and biological duplicates. RNA fold enrichment after IP was calculated as $2^{(\text{No RT} - \text{RT})}$, where No RT is the reaction performed without reverse transcriptase and RT is the reaction with reverse transcriptase. Normalized mRNA levels between carbon sources were calculated as $2^{(\text{Galactose IP} - \text{Dextrose IP})}$, then multiplied by the normalized protein levels after IP.

TABLE 1.3. Strains used in this study

Description	Strain	Genotype	Source
Wild-type	yWO7	MAT α , <i>leu2-3</i> , <i>ura3-52</i> , <i>rpb1-1</i>	Caponigro <i>et al.</i> , 1993
<i>puf1</i> Δ	yWO102	MAT α , <i>leu2-3</i> , <i>trp1-1</i> , <i>ura3-52</i> , <i>cup1::LEU2/PM</i> , <i>puf1::NEO</i>	Ulbricht and Olivas, 2008
<i>puf2</i> Δ	yWO48	MAT α , <i>his4-539</i> , <i>leu2-3</i> , <i>ura3-52</i> , <i>rpb1-1</i> , <i>puf2::URA3</i>	Ulbricht and Olivas, 2008
<i>puf3</i> Δ	yWO43	MAT α , <i>his4-539</i> , <i>leu2-3</i> , <i>trp1-1</i> , <i>ura3-52</i> , <i>rpb1-1</i> , <i>cup1::LEU2/PM</i> , <i>puf3::NEO</i>	Olivas and Parker, 2000
<i>puf4</i> Δ	yWO105	MAT α , <i>his4-539</i> , <i>lys2-201</i> , <i>ura3-52</i> , <i>rpb1-1</i> , <i>puf4::LYS2</i>	Ulbricht and Olivas, 2008
<i>puf5</i> Δ	yWO49	MAT α , <i>leu2-3</i> , <i>trp1-1</i> , <i>ura3-52</i> , <i>rpb1-1</i> , <i>puf5::URA3</i>	Ulbricht and Olivas, 2008
<i>PUF5-GFP*</i>	yWO199	MAT α , <i>his3Δ1</i> , <i>leu2Δ0</i> , <i>met15Δ0</i> , <i>ura3Δ0</i> , <i>PUF5::GFP-HIS3MX</i>	Huh <i>et al.</i> , 2003
<i>PUF1-GFP*</i>	yWO200	MAT α , <i>his3Δ1</i> , <i>leu2Δ0</i> , <i>met15Δ0</i> , <i>ura3Δ0</i> , <i>PUF1::GFP-HIS3MX</i>	Huh <i>et al.</i> , 2003
<i>PUF4-GFP*</i>	yWO203	MAT α , <i>his3Δ1</i> , <i>leu2Δ0</i> , <i>met15Δ0</i> , <i>ura3Δ0</i> , <i>PUF4::GFP-HIS3MX</i>	Huh <i>et al.</i> , 2003
Δ <i>puf1-5</i>	yWO204	MAT α , <i>his4-539</i> , <i>leu2-3</i> , <i>lys2-201</i> , <i>trp1-1</i> , <i>ura3-52</i> , <i>rpb1-1</i> , <i>puf1::NEO</i> , <i>puf2::TRP1</i> , <i>puf3::NEO</i> , <i>puf4::LYS2</i> , <i>puf5::URA3</i>	Ulbricht and Olivas, 2008
Δ <i>puf1-5</i>	yWO268	MAT α , <i>his4-539</i> , <i>leu2-3</i> , <i>lys2-201</i> , <i>trp1-1</i> , <i>ura3-52</i> , <i>rpb1-1</i> , <i>puf1::NEO</i> , <i>puf2::TRP1</i> , <i>puf3::NEO</i> , <i>puf4::LYS2</i> , <i>puf5::URA3</i> , <i>ura3::KanMX3</i>	This study
<i>yhb1</i> Δ	yWO269	MAT α , <i>leu2-3</i> , <i>rpb1-1</i> , <i>ura3-52</i> , <i>yhb1::URA3</i>	This study
<i>PUF1-TAP*</i>	yWO286	MAT α , <i>his3Δ1</i> , <i>leu2Δ0</i> , <i>met15Δ0</i> , <i>ura3Δ0</i> , <i>PUF1::TAP-HIS3MX</i>	Ghaemmaghami <i>et al.</i> , 2003
<i>PUF4-TAP*</i>	yWO287	MAT α , <i>his3Δ1</i> , <i>leu2Δ0</i> , <i>met15Δ0</i> , <i>ura3Δ0</i> , <i>PUF4::TAP-HIS3MX</i>	Ghaemmaghami <i>et al.</i> , 2003
<i>PUF5-TAP*</i>	yWO288	MAT α , <i>his3Δ1</i> , <i>leu2Δ0</i> , <i>met15Δ0</i> , <i>ura3Δ0</i> , <i>PUF5::TAP-HIS3MX</i>	Ghaemmaghami <i>et al.</i> , 2003

* The *PUF-GFP* and *PUF-TAP* strains contain carboxy-terminal, in-frame insertions of either the GFP gene or TAP sequence to a PUF gene

TABLE 2.3. Plasmids used in this study

Plasmid	Description	Marker(s)	Source
pWO14	pG-1/ <i>PUF3RD</i>	Amp ^R , <i>TRP1</i>	Houshmandi and Olivas, 2005
pWO15	pG-1 derivative/FLAG tag	Amp ^R , <i>URA3</i> , <i>TRP1</i>	Schena and Yamamoto, 1988
pWO16	FLAG- <i>PUF3RD</i>	Amp ^R , <i>URA3</i> , <i>TRP1</i>	This study
pWO18	pGEX6p-3/GST- <i>PUF5</i>	Amp ^R	Jackson <i>et al.</i> , 2004
pWO48	pBS/ <i>PUF1RD</i>	Amp ^R	Ulbricht and Olivas, 2008
pWO58	<i>LEU2</i> , CEN Vector	Amp ^R , <i>LEU2</i>	Brachmann <i>et al.</i> , 1998
pWO61	pGAL- <i>MFA2pG</i>	Amp ^R , <i>LEU2</i>	Ulbricht and Olivas, 2008
pWO100	pGAL- <i>PGK1/HXK1</i> 3'UTR	Amp ^R , <i>URA3</i>	Ulbricht and Olivas, 2008
pWO102	pGAL- <i>PGK1</i>	Amp ^R , <i>URA3</i>	Ulbricht and Olivas, 2008
pWO114	FLAG- <i>PUF1</i>	Amp ^R , <i>URA3</i> , <i>TRP1</i>	This study
pWO115	FLAG- <i>PUF1RD</i>	Amp ^R , <i>URA3</i> , <i>TRP1</i>	This study
pWO116	pRS415- <i>GPD-PUF4</i> /CEN	Amp ^R , <i>LEU2</i>	Hook <i>et al.</i> , 2007
pWO127	pGAL- <i>PGK1/YHB1</i> 3'UTR	Amp ^R , <i>URA3</i>	This study
pWO128	pGAL- <i>PGK1/YHB1</i> 3'UTR	Amp ^R , <i>LEU2</i>	This study
pWO129	pGAL- <i>PGK1/yhb1-2</i> 3'UTR	Amp ^R , <i>URA3</i>	This study
pWO162	<i>URA3</i> → <i>LEU2</i> marker swap	Amp ^R	ATCC #87552
pWO183	<i>URA3</i> → <i>KanMX3</i> marker swap	Amp ^R	Voth <i>et al.</i> , 2003
pWO192	FLAG- <i>PUF2RD</i>	Amp ^R , <i>URA3</i> , <i>TRP1</i>	This study
pWO193	FLAG- <i>PUF4RD</i>	Amp ^R , <i>URA3</i> , <i>TRP1</i>	This study
pWO194	FLAG- <i>PUF4</i>	Amp ^R , <i>URA3</i> , <i>TRP1</i>	This study
pWO195	FLAG- <i>PUF5RD</i>	Amp ^R , <i>URA3</i> , <i>TRP1</i>	This study
pWO196	FLAG- <i>PUF5</i>	Amp ^R , <i>URA3</i> , <i>TRP1</i>	This study
pWO197	pGAL- <i>PGK1/yhb1-1</i> 3'UTR	Amp ^R , <i>URA3</i>	This study
pWO199	pGAL- <i>PGK1/yhb1-1</i> 3'UTR	Amp ^R , <i>LEU2</i>	This study
pWO200	FLAG- <i>PUF5RDmut</i>	Amp ^R , <i>URA3</i> , <i>TRP1</i>	This study
pWO201	pGEX6p-3/GST- <i>PUF1</i>	Amp ^R	This study
pWO202	pGEX6p-3/GST- <i>PUF4</i>	Amp ^R	This study

TABLE 3.3. Oligonucleotides used in this study

Oligo	Description	Sequence
oWO77	<i>YHB1</i> Δ up primer	tttaccattttcaacaacacacaaagactttatcatgacatgattacgccagctcg
oWO78	<i>YHB1</i> Δ down primer	aatcagtaataaaattgaagttccgaggctaacgcctacgactcactatagggctaattggg
oWO105	<i>HXK1</i> probe	cataaggcgcactcactcataag
oWO145	<i>PUF1RD</i> down primer	cgggatccgatggcaattccgatgaatacacaatcaattcg
oWO159	<i>YHB1</i> probe	cgctaaactgcacggttgac
oWO238	<i>MFA2</i> probe	atattgattagatcaggaattcc
oWO262	<i>YHB1</i> 3'UTR up primer	ggcagatctgtcaaccgtgcaagtttag
oWO263	<i>YHB1</i> 3'UTR down primer	ccgaagcttgcttccatgacaggttccg
oWO447	<i>PGK1</i> probe	ccaaagaagcaccaccacagtagag
oWO466	<i>PUF1</i> up primer	cgggatccgatggataaaagtaagcagatgaacatc
oWO467	<i>PUF1</i> down primer	acgcgtcgacggcgccgcttccctgtagttggacac
oWO468	<i>PUF1RD</i> up primer	cgggatccgatggcaattccgatgaatacacaatcaattcg
oWO480	<i>yhb1-2</i> 3'UTR up primer	gtggaaatattagatagtaagtaaagattgacaacaattataagatgaataagcgccag
oWO481	<i>yhb1-2</i> 3'UTR down primer	ctggcgcttattcatcttataaattgttgcaatctttacttactatctaataattccac
oWO513	<i>yhb1-1</i> 3'UTR up primer	gtggaaatattagatagtaagtaaagattgactatgtattataagatgaataagcgccag
oWO514	<i>yhb1-1</i> 3'UTR down primer	ctggcgcttattcatcttataaatacatagtaactttacttactatctaataattccac
oWO568	<i>PUF5RD</i> down primer	gtagagacatgggagatccggatcattacttatctaggtcgacctattttctgtgtaatcgttgaag
oWO569	<i>PUF5RD</i> up primer	tctcgagctcccattggactacaaggacgacgatgacaaggatccggagttgatctggaatggatggt
oWO570	<i>PUF2RD</i> down primer	gagtctaggtcgacggaaacagaaacgcctctggc
oWO571	<i>PUF2RD</i> up primer	gatgcggatccgcctccaccatcattatcggtatg
oWO610	<i>PUF4</i> up primer	tctcgagctcccattggactacaaggacgacgatgacaaggatccgatgtctacaaaagggttgaaagaaga
oWO611	<i>PUF4</i> down primer	gtagagacatgggagatccggatcattacttatctaggtcgacctatgaattaaatgtaacattcc
oWO612	<i>PUF5</i> up primer	tctcgagctcccattggactacaaggacgacgatgacaaggatccgatgatcaataacgaaccatttcca
oWO613	<i>PUF5</i> down primer	gtagagacatgggagatccggatcattacttatctaggtcgacttatggcaagtgaaatgaagttc
oWO638	<i>PUF4RD</i> up primer	tctcgagctcccattggactacaaggacgacgatgacaaggatccgaataataacagtaagtctacc
oWO790	<i>CBS1</i> qPCR up primer	cgagctcgagtagtgaacatggac
oWO791	<i>CBS1</i> qPCR down primer	cggcaaatggcaggtattcgc
oWO854	<i>YHB1</i> qPCR up primer	agcaaatggccacaagcatcg
oWO855	<i>YHB1</i> qPCR down primer	accaacgattggataatgctcagg
oWO861	<i>HXK1</i> qPCR up primer	tttaaggaagccgctgtaag
oWO862	<i>HXK1</i> qPCR down primer	cttgcgtcaccagtcctccatag

oWO865	GST- <i>PUF1</i> up primer	gcggatccatggataaaagtaagcagatgaac
oWO866	GST- <i>PUF1</i> down primer	tagcggccgctcagttgcagagtgtaaagtc
oWO867	GST- <i>PUF4</i> up primer	ccgaattccatgtctacaaaaggtttgaaagaag
oWO868	GST- <i>PUF4</i> down primer	tagcggccgcctatgaatctaaatgtaacattccg
oWO898	<i>YHB1</i> WT PRE	guaaagauuguguauguauuuauaagaugaaua
oWO899	<i>yhb1-1</i> mutant PRE	guaaagauugacuauguauuuauaagaugaaua
oWO900	<i>yhb1-2</i> mutant PRE	guaaagauugacaaacaauuuauaagaugaaua

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**CHAPTER 4: PUMILIO PROTEINS REGULATE mRNAs ASSOCIATED
WITH THE PROGRESSION OF PARKINSON'S DISEASE**

INTRODUCTION

Parkinson's disease (PD) is the most common motor system disorder and is caused by the loss of dopamine-producing neurons. The decreased levels of the neurotransmitter dopamine, which is required for movement coordination and balance, leads to tremors, slowness of movements, rigidity, and impaired balance (Bueler, 2009). Within neural cells, Parkinson's is characterized by aggregation of the α -synuclein protein (encoded by the gene SNCA), and this aggregation impairs mitochondrial function. In fact, many genes that are mutated or aberrantly expressed in PD patients are involved in maintaining different aspects of proper mitochondrial function. It is hypothesized that the loss of dopamine-producing neurons in familial and sporadic PD may largely be due to mitochondrial dysfunction (Bueler, 2009). Coding mutations have been identified in several PD-associated genes, yet it is often the over-expression of these genes that is implicated in the disease. For example, increased expression of SNCA is correlated with increased severity and earlier onset of PD, presumably due to the increased levels of α -synuclein protein that promote aggregation (Wood-Kaczmar *et al.*, 2006). Aberrantly high expression of the kinase LRRK2 is also linked to both familial and sporadic Parkinson's cases and causes mitochondrial dysfunction and apoptosis (Bueler, 2009); however, the molecular mechanisms that cause aberrant expression of these genes are not well understood.

In addition to the well-studied transcriptional regulation of genes, post-transcriptional regulation of mRNA stability and translation are increasingly acknowledged as key steps in gene expression control. Typically, control elements

located in the 3' untranslated region (UTR) of mRNAs recruit microRNA (miRNA) complexes or regulatory proteins that influence mRNA decay and/or translation rates. One important family of 3' UTR regulatory proteins is the Puf family, with multiple members in mammals, insects, worms, plants and yeast. Pumilio from *Drosophila melanogaster* (DmPum) and FBF from *Caenorhabditis elegans* were the founding members of this group, thus providing the Puf family name. Puf proteins regulate diverse processes such as cell development, stem cell maintenance, and organelle biogenesis by binding to specific sequences of targeted mRNAs and stimulating mRNA degradation and suppressing translation (Miller and Olivas, 2011). Puf proteins are also involved in several aspects of neural function, such as neuronal excitability (Schweers *et al.*, 2002), dendrite morphogenesis in peripheral neurons (Ye *et al.*, 2004), and synaptic growth and plasticity at the neuromuscular junction (Menon *et al.*, 2009; Marrero *et al.*, 2011). Puf expression increases during long-term memory formation, and its disruption results in defective memory (Dubnau *et al.*, 2003). In humans, the Puf proteins Pum1 and Pum2 are expressed widely, including in stem cells and brain tissue (Spasov and Jurecic, 2002). Microarray studies indicate that Pum1 levels decrease ~2-fold in some PD patient brain tissue, while Pum2 levels decrease ~1.3 fold (Sutherland *et al.*, 2009); however, there is little known about which mRNAs are regulated by Pufs in humans that may link Puf function with neural activity and PD. At the molecular level, Puf proteins directly elicit translation initiation repression through protein interactions that inhibit cap-binding events, or stimulate deadenylation and decapping steps of decay through interactions with mRNA decay machinery (Miller and Olivas, 2011). Recently, Puf

proteins have also been found to act cooperatively with the miRNA regulatory system. Global studies to identify mRNAs bound by human Pum1 and Pum2 determined that 3' UTRs containing Puf binding sites are enriched in miRNA binding sites (Galgano *et al.*, 2008). It was hypothesized that binding of Puf proteins to a 3' UTR may alter the secondary structure of the 3' UTR such that miRNA sites become more accessible and therefore more efficient in RNA repression. Specifically, miRNA sites located near Puf binding sites are often located in highly structured 3' UTR regions that are poorly accessible (Incarnato *et al.*, 2013), and the distance between the miRNA and Puf sites is typically less than 50 nt (Jiang *et al.*, 2013). For example, Puf binding sites are often associated with miR-410 target sites located in highly structured regions (Leibovich *et al.*, 2010). Experimentally, binding of human Pum1 to the 3' UTR of the p27 tumor suppressor mRNA altered the local RNA structure and permit binding of miR-221 and miR-222 for RNA repression (Kedde *et al.*, 2010). Moreover, Pum1 RNA-binding activity to the p27 3' UTR was dependent on phosphorylation of Pum1 in response to growth factor stimulation (Kedde *et al.*, 2010). Binding of human Puf proteins to the 3' UTR of the E2F3 oncogene also enhanced miRNA-mediated repression of this mRNA, and several types of cancer circumvent miRNA regulation by shortening the 3' UTR, thereby eliminating the Puf binding site (Miles *et al.*, 2012). In addition to making miRNA sites more accessible, Pufs may also work coordinately with miRNAs through interactions with the miRNA complex protein Argonaute, and these interactions inhibit translation elongation (Friend *et al.*, 2012).

The miRNA regulatory system is involved in many diseases, and Parkinson's is no exception. Elimination of miRNAs in both *Drosophila* and mice results in a loss of dopamine neurons and motor defects similar to PD (Mouradian, 2012). The SNCA mRNA, which contains a highly conserved 3' UTR, is repressed by at least two miRNAs, miR-7 and miR-153 (Mouradian, 2012). The LRRK2 mRNA is repressed by miRNA-205, and this miRNA is downregulated in patients with PD (Cho *et al.*, 2013). Bioinformatic analysis revealed that many of the known PD-associated genes contain miRNA binding sites in their 3' UTRs (Figure 4.1). Moreover, we determined that many of these 3' UTRs also contain perfect or near perfect Puf binding sites (UGUA(A/U)AUA) (Figure 4.1). One of the Puf binding sites in SAT1 has been shown to be clinically relevant, as a deletion of the UGU was identified as a common mutation in Parkinson's patients (Lewandowski *et al.*, 2010). Given the cooperative activity of Puf proteins and miRNAs in repressing mRNA targets, and the knowledge that many PD genes are aberrantly upregulated in the disease state, we hypothesized that Puf proteins bind and regulate multiple mRNA targets involved in Parkinson's disease. Further support for this hypothesis comes from the global study identifying mRNAs physically associated with human Pufs, where the SNCA mRNA was pulled down with Pum1 (Galgano *et al.*, 2008). Since many PD-associated genes are involved in proper mitochondrial function, Pufs may play a global role in regulating mitochondrial function in humans. Such a role may be conserved across species, as the yeast Puf3 protein regulates many genes required for mitochondrial function (Olivas and Parker, 2000; Gerber *et al.*, 2004; Foat *et al.*,

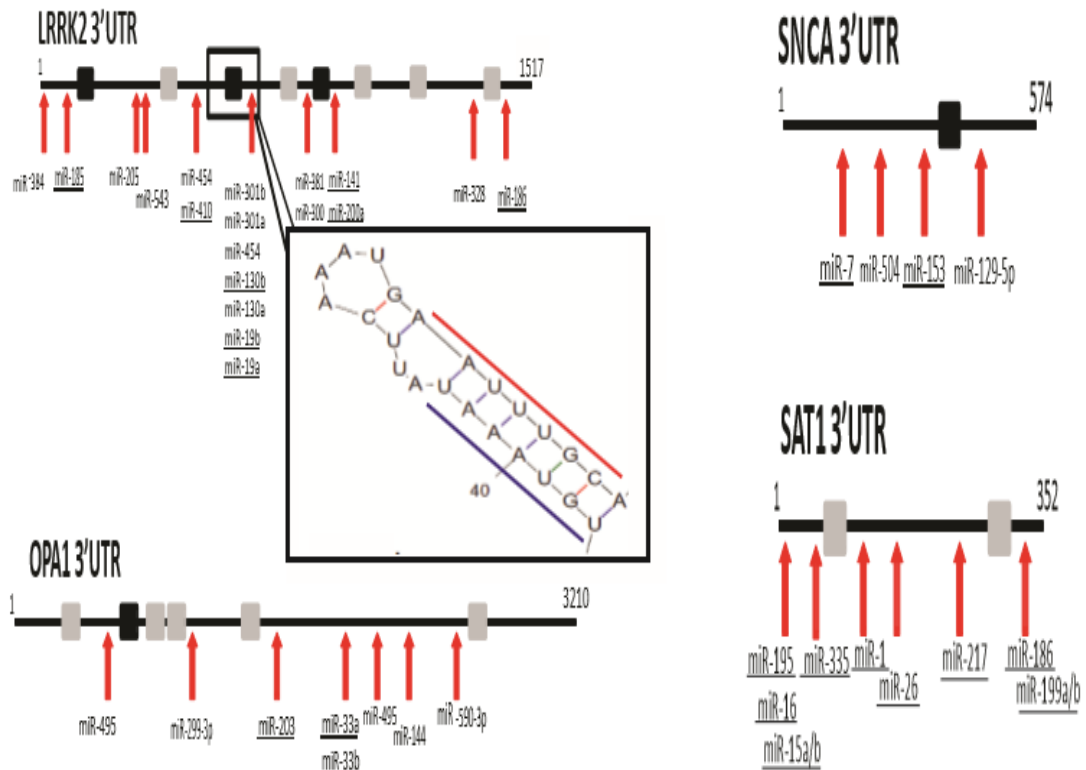


Figure 4.1. Bioinformatic analysis of potential Puf binding sites and miRNA binding sites in the 3' UTRs of Parkinson's associated genes. Lines represent 3' UTR regions, not drawn to scale. Perfect Puf binding sequences UGUA(A/U)AUA are shown as filled black boxes and imperfect Puf binding sequences (contains the core UGU with variable A/U rich downstream nucleotides) are shown as filled in grey boxes. miRNA binding sites are noted by red arrows, with miRNAs known to be expressed in SH-SY5Y neural cells underlined. A predicted secondary structure example between Puf and miRNA binding site regions is shown in the open boxes, with the Puf site lined in blue and the miRNA site lined in red.

2005). Herein, I describe key findings that implicate Pufs in regulating PD-associated mRNAs, thereby advancing both the Pumilio field and the Parkinson's disease field. Also, I have established the use of human cell lines as model organisms in the Olivas Lab for studying Pumilio function during PD progression. The completed and future research presented herein will significantly advance the field of Parkinson's disease by revealing the extent to which Puf proteins are directly involved in coordinately regulating several mRNAs involved in PD and mitochondria. Also, valuable information on the interplay between miRNA and Puf-mediated repression of these mRNAs will be discussed, as well as the mechanisms by which Puf proteins may be inactivated under PD conditions to cause upregulation of the target mRNAs. The knowledge gained from these studies will provide a new line of inquiry into Pufs as therapeutic targets for Parkinson's disease treatment.

MATERIALS AND METHODS

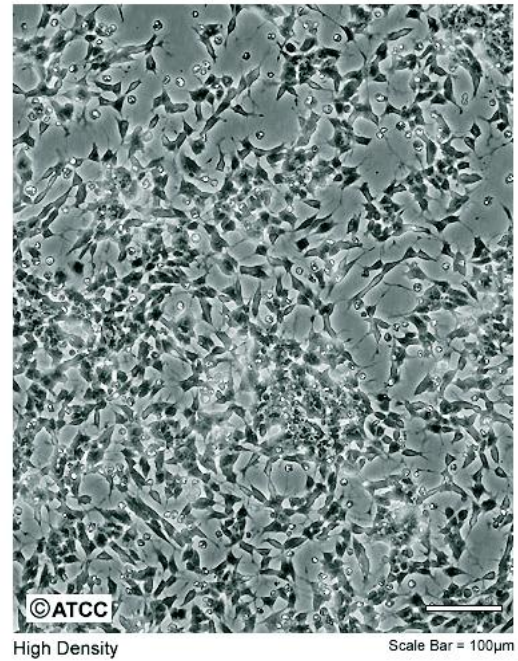
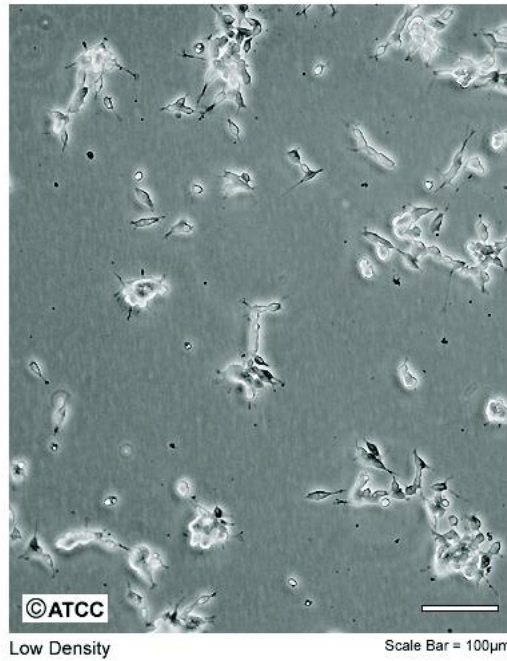
Plasmids and Oligos Used in this Study

All oligos used in this study are listed in Table 2.4. All plasmids created and used in this study are listed in Table 3.4. qPCR primers were all designed using quantprime software. All plasmids were prepared using the Promega Pure-Yield kit to ensure quality during transfection and other downstream applications. Renilla luciferase reporter constructs were made by standard restriction site cloning of 3' UTR regions PCR-amplified from SH-SY5Y cDNA. Standard site-directed mutagenesis protocols were used in the creation of mutant reporter constructs.

Cell lines Information and Medium for Growth

Two different cell lines were used to perform the studies presented herein. The neuroblastoma human cell line SH-SY5Y and the human embryonic kidney cell line HEK-293 were used. The SH-SY5Y cells were purchased from ATCC, and detailed information for this cell line can be found on ATCC's website. Briefly, these cells are from a 4 year old female brain with metastatic bone cancer. The growth properties are described as mixed. This term means that the cell line is both suspension and adherent when cultured in flasks. Specifically, when visualizing these cells in the growth flask under a microscope, some cells will appear stuck to the plastic while others will be floating around in suspension. Neurites/dendrites are observable as arms extending from the cells. These cells also tend to clump/cluster when they become adherent. Both individual cells as well as large aggregates of cells are observable (Figure 4.2). Renewal of the growth medium is required every 4-7 days and passage is typical every 10-14 days at a 1:10 ratio. The SH-SY5Y cells exhibit moderate levels of dopamine beta hydroxylase activity and have a reported saturation density greater than 1×10^6 cells/sq cm. The base cell medium used for this cell line is a 1:1 mixture of ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003, and Ham's F12 Medium (DMEM 1:1 with F-12). This medium is then supplemented with 10% Fetal Bovine Serum (HyClone FBS) and 5% Penicillin/ Streptomycin antibiotic mixture (HyClone Penicillin-Streptomycin). The HEK-293 cells were acquired from the Nichols lab in the Chemistry department at UMSL. These cells exhibit an adherent growth phenotype (Figure 4.2). Medium renewal is required every 2-3 days and passaging is performed every 7 days at

A.



B.

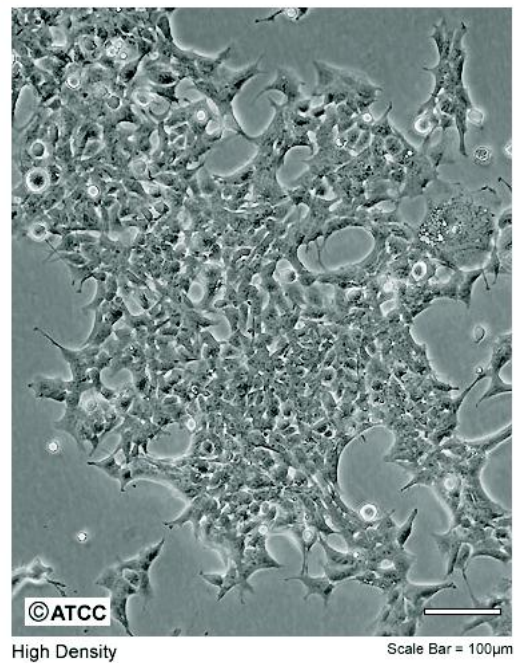
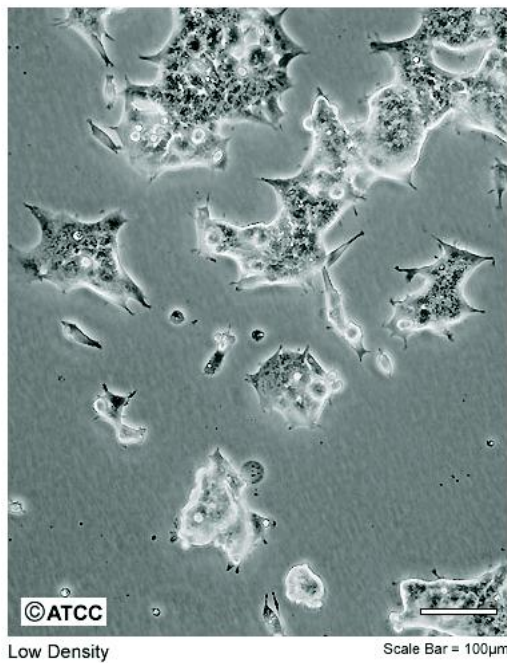


Figure 4.2. Examples of cells used in this study. (A) Shown are SH-SY5Y human neuroblastoma cells at low density (left panel) and at high density (right panel). Images are modified from the ATCC website that the cells were purchased from. (B) Shown are HEK-293 human embryonic kidney cells at low density (left panel) and at high density (right panel). Images are modified from the ATCC website that the cells were purchased from.

a 1:10 ratio. The medium for this cell line is Eagle's DMEM supplemented with 10% FBS and 5% Penicillin/Streptomycin.

For basic cell growth and maintenance, the following disposable items are required. Corning culture flasks are used to maintain cell lines. Specifically, canted, 75cm² vent cap flasks were used (10-126-37 Fisher). In addition, disposable, individually wrapped serological pipettes in sizes 5ml, 10ml and 25ml are required. Nalgene MF75 sterile filter units (500mL, 0.2µm, aPES, 90mm) are needed for filtering of media. Other required plastics include sterile nalgene cryogenic vials for stocking (2mL), conical tubes (15mL and 50mL), and 96 well plates in both clear (Costar, CLS3595) and white (Costar, CLS3362) for downstream assays.

Medium Preparation

Sterile preparation of media is required before thawing cells for use. Media preparation, cell culturing, and most experiments are done in sterile hoods in the cell culture facility. Before preparing media, gloves are put on and sprayed with 70% ethanol for sterility. After gloves are sterile, thoroughly wipe down the interior of the hood with 70% ethanol and chemwipes to ensure a sterile working area. Sterility under the hood is key to maintaining healthy, contamination-free cells. Media is purchased in either 1 liter or 500mL volumes. FBS is purchased as a 500mL volume and should be thawed and aliquoted into 50mL sterile conical tubes for use in media preparation. Freeze unused conicals for future use (-20°C). Penicillin/Streptomycin arrives as a 100 mL volume and should be aliquoted into 5mL portions in sterile 15mL conical tubes. Freeze remaining conicals for later use. Using an MF75 500mL sterile filter, add 445mL

of base medium, 50 mL of pre-thawed FBS, and 5mL of pre-thawed Pen/Strep and vacuum filter under the sterile hood in the cell culture facility. Once filtered, immediately cap bottle and seal with parafilm. The media is now ready for use in culturing cells. The media is stored in the refrigerator (4°C) until ready for use. It is a good idea to always pay attention to how much media you have remaining and to always have media made before getting new cells or passaging currently growing cells.

Cell Expansion

After media has been made, cells can be thawed and expanded for experimental use. First wipe down the hood with 70% ethanol ensuring a sterile working environment. Next, remove previously made media from the 4° fridge, place in the 37°C bath and allow to warm so as to not shock the cells (15 minutes at 37°C is sufficient). Once the media has been warmed, wipe the entirety of the bottle with 70% ethanol. Place the bottle in the hood and wipe further around the cap with 70% ethanol. Remove the parafilm around the cap and continue to wipe with 70% ethanol as you remove the cap being sure to get the threading. Now your media is ready for use. It is a good idea to prepare all other equipment needed for cell culture work at this time. This includes wiping down the pipettes and pipette aid that will be used, being sure to wipe down the single use 5mL, 10mL and 25mL pipettes in their plastic wrapping (i.e. before you unwrap them). Furthermore, you should label a 75mm flask with the cell type and passage number of the cells you wish to expand. Thorough cleaning of the flask should be done, and once placed under the hood, the threads should be cleaned as the cap is removed. Always using sterile pipettes, aliquot the desired amount of media into the

flask, in most situations it will be 9mLs of media to 1mL of cells (either from freezer stock or passaged cells). Once the media is in the flask, you can get a vial of cells from the liquid nitrogen chamber. The current liquid nitrogen storage sheet is on the computer in the cell culture facility. With sterile gloves, remove one vial of cells to be thawed and expanded. Note that the tube should describe the cell line and provide the passage number for you to write on the flask if the information was not previously known. Using forceps dip the vial into the 37°C water bath and allow the cells to thaw being sure not to fully submerge the vial or expose the cap to the bath. Once the cells have thawed, dip the vial in 70% ethanol several times to sterilize. Place 9mLs of media into a 15mL conical tube and add the 1mL of cells from the vial. Note, any steps involving cells are done under the hood when possible. Spin the 15mL conical tube at 125xG for 5-7 minutes. This step allows for the removal of DMSO used in stocking the cell line. After centrifugation, a pellet of cells should be visible in the conical tube. Using the vacuum under the hood and a glass pipette (9 inch), carefully aspirate the media from the cell pellet. Resuspend the pellet with 1mL of fresh media and add to the flask containing 9mL of media for a total of 10mL. This provides the 1:10 dilution required for both cell lines. Other cell lines can differ and should be assessed accordingly. The total cells in stocked vials should be $\sim 1.6 \times 10^6$. View the cells under a microscope to ensure their presence and then place the flask in the 37° dry incubator for growth. Check the cells every day or two as growth should become visibly evident under the microscope. Also, growth media contains a pH indicator and over time the

media becomes more acidic, causing a change in the media color from pink to yellow. This is a good sign of healthy growing cells.

Cell Counting, feeding and Passaging

As the cells continue to grow they will eventually reach a growth density that is ready for experiments or passaging. The common term used to describe cell growth is confluency. This term describes how much area of the flask the cells seem to cover when viewed under a microscope. For example, 70% confluency means that about 70% of the visible area of the flask is covered with cells, leaving 30% of the bottom of the flask visible. The rate at which cell confluency increases is dependent on the cell line. SH-SY5Y cells can take 10 days or more to reach 70% confluency, while HEK-293 cells can reach 70% confluency in as little as 5 days. During the growth of the cells the media may need to be switched out. This is termed feeding the cells. Growth media will change from pink to yellow when feeding is required, or check the information available about the cell line for how often to feed. Before feeding, be sure to warm fresh media in the 37° incubator. Remove the flask and aspirate the old growth media into a 15mL conical and save if the cell line is suspension. Immediately pipette new media onto the adherent cells as they will dry quickly. Spin down the suspension cells as before and resuspend in fresh media. Combine the resuspended suspension cells into the flask with the adherent cells and return to the incubator.

Once the cells have reached 70-75% confluency they are ready to be passaged. Again, retrieve and sterilize all equipment needed for passaging, including a new flask labeled with the new passage number. Also at this time, you should have warmed your

growth media as well as the trypsin 1X (0.25%) in HBSS with EDTA. Trypsin is used to remove adherent cells from the flask for counting and passage. While working under the hood, aspirate the growth media into 15mL conical tubes. Note, if your cells are suspension based, this media will be saved and used because it contains cells (SH-SY5Y cells); however, if your cells are strictly adherent (HEK-293 cells) this media can be disposed of in the proper receptacle. Ensure that all the media is removed from the cells, as growth medium inhibits trypsin activity. Some people choose to wash the cells with PBS to ensure removal of the growth medium; however, I have not found this to be necessary. Next, apply 1.5-2mLs of trypsin to the cells in the flask, being sure to cover the entirety of the cells. Place the flask in the 37° incubator for 5 minutes, checking every two minutes to ensure trypsin activity. The cells on the bottom should become visibly removed upon inspection with the naked eye. After 5 minutes, all the cells should appear to be removed from the bottom of the flask. Return the flask to the hood and add media to a final volume of 10mLs to inactivate the trypsin. Pipette the cells and medium into 15mL conical tubes. You should now have adherent and suspension cells (if using SH-SY5Y cells) in 15mL conical tubes. Spin tubes at 125xG for 10 minutes. A cell pellet should be visible in all tubes. Aspirate the media from the pellets and resuspend the pellet in 5mLs of fresh media. Now the cells are ready to be counted. Using the cell counter, perform a cell count by pipetting 20µL onto a compatible slide. Also, at this time viability of the cells can be checked by diluting the cells 1:1 with trypan blue and then performing a cell count. Be sure to change the dilution factor to 2 to account for the dilution. A percentage of viable cells will be shown after the count is completed.

Manual adjustment is sometimes necessary when the software misses counts. Be sure to check this each time you perform a cell count. The cell count will be variable, so you must adjust your final cell concentration to be near the 1×10^6 cells per mL for passage. After adjusting your cell concentration, you can now add 1mL of cells to the 9mLs of fresh media in the new flask, successfully passaging the cells. At this point, remaining cells can be used for experiments or for stocking of more cells. It is also important to note the maximum passage number to use the cells during experiments. Depending on the experiment and observed growth, this is usually at the experimenter's discretion.

Stocking Cells

In order to stock cells, media must be made containing all the components previously described and an additional 5% DMSO. This will be a separate media used exclusively for stocking cells. Once cells have been counted (should be $\sim 1.5 \times 10^6$ cells/mL), spin down again for 10 minutes at 125xG. Remove the media leaving the cell pellet behind and resuspend with the same volume of media now containing 5% DMSO. Aliquot 1mL of cells into cryogenic vials labeled with cell type, passage number and date. To ensure viability the cells must be frozen slowly overnight. To do so, use the vial freezing holders (isopropanol chamber) in the cell culture facility. These allow for the addition of isopropanol to the bottom layer to evenly freeze the cells. The cells are placed in the holder and inserted into the -80°C freezer overnight. The following day, vials can be arranged in a freezer box in the liquid nitrogen vessel for later use.

Pumilio1 and Pumilio2 Knockdown

For knockdown in SH-SY5Y cells, special modified siRNA “SMARTpools” were ordered from Thermo Scientific Dharmacon. Five nmol of Accell Human Pum1 (9698) siRNA SMARTpool and Accell Human Pum2 (23369) siRNA SMARTpool was purchased. The sequences targeted by the SMARTpool for Pum1 and Pum2 are listed in Table 1.4. Upon arrival, the 5x siRNA buffer was diluted to 1X using RNase/Dnase-free water. The 5nmol of siRNA was resuspended in 50 μ L of 1X siRNA buffer to a final concentration of 100 μ M and stored at -80°C in 4 μ L aliquots for use in knockdowns. Once cells reached 70-75% confluency, they were trypsin treated and counted. 10,000 cells were plated per well in a 96 well cell culture ready plate and allowed to adhere overnight. The next day, the accell delivery media (provided with SMARTpool siRNAs) was warmed to 37°C for use in the transfection. Remove the growth media by careful pipetting. Cells should be adherent to the well and visible with the naked eye under a microscope. Disregard non-adherent cells at this step. Immediately add 99 μ L of accell delivery media to the wells. Do not let wells sit without media for long as cells will dry out and die. Next, add 1 μ L of the 100 μ M siRNA SMARTpool to the well. If knocking down Pum1 and Pum2 reduce growth media to 98 μ L and add 1 μ L of each SMARTpool. Use scrambled siRNAs as a negative control. Return 96-well plate to the incubator and allow to sit for 48-72 hours before assaying. For maximum knockdown, perform a second “hit” of siRNAs 24 hours after the first hit. Simply add an additional 1 μ L of SMARTpool siRNA and return to the incubator. Perform all assays 72 hours after the first siRNA application.

For knockdown in HEK-293 cells a different protocol was used. Silencer Pre-designed siRNAs against PUM1 (AM16708) and PUM2 (4392420) were purchased from Ambion. The sequences of the siRNAs are listed in Table 1.4. Each 5nmole siRNA was resuspended in RNase/Dnase-free water to a final concentration of 5 μ M. The working concentration for knockdown is 5nM. Genemute transfection agent was used to facilitate knockdown of Pumilio proteins as this reagent has been optimized for delivery of siRNAs. The following example shows how to set up Pum1 and Pum2 knockdown in a 96 well format. The day before knockdown is performed, plate 10,000 cells per well as described previously. Create a cocktail for knockdown in each individual well by combining 10 μ L of Pum1 siRNA (5 μ M), 10 μ L of Pum2 siRNA (5 μ M), 10 μ L of 5X genemute reaction buffer, and 20 μ L RNase/DNase-free water. This cocktail now has a 1 μ M concentration of each Pum. Utilizing 0.5 μ L of the cocktail into the 100 μ L volume in the well will provide the 5nM concentration for quality knockdown. To do this, combine 0.5 μ L of the cocktail with 0.3 μ L of genemute for each well that will have knockdowns. For example, if you need to do 20 wells combine 10 μ L of cocktail with 6 μ L of genemute (see instructions for siRNA:genemute ratios and trouble shooting). Allow the complex to sit for 10-15 minutes to fully form transfection ready complexes. During this time, remove media from wells and replace with 99.2 μ L of fresh media, allowing 0.8 μ L of room for the transfection complex. After 10-15 minutes, apply 0.8 μ L of transfection complex to each well, raising the final volume to 100 μ L per well and a final siRNA concentration of 5nM each. Allow to sit for 24-72 hours before assaying knockdown.

Optimal knockdown is typically observed after 48 hours and still very good at 72 hours post transfection.

Quantitative PCR

qPCR was used to verify knockdown and to evaluate expression of target genes in response to knockdown. 48-72 hours post-transfection of knockdown siRNAs, total RNA was prepared using the Direct-Zol RNA preparation kit (Zymo Research). 300µL of trizol was added to each well for a total volume of 400µL. The cells were subjected to aggressive pipetting to remove cells from wells. The 400µL of sample was then moved to microcentrifuge tubes and total RNA was extracted as described by the manufacturer. Total RNA was not DNase treated in column; however, DNase treatment was done following total RNA preparation using the turbo DNase kit purchased from Ambion. Total RNA was quantified using the nanodrop system. 500ng of total RNA was used to create cDNA using Biorad iScript Reverse Transcription Supermix (170-8840) in accordance with the protocol. For each qPCR reaction, 2µL of each primer (100ng) was mixed with, 3.5µL of diluted cDNA (5X or 10X dilution) and 7.5µL of SSO Advanced Sybr Green (BioRad). qPCR was performed on a BioRad CFX 96 real-time system. Cycle parameters are as follows: Initial denature step 95°C for 3 minutes, denature 95°C for 10 seconds, anneal at 52°C for 10 seconds, and extend at 72°C for 30 seconds. Data is collected after each extension for a total of 40 cycles.

Protein Analysis

Total protein was prepared immediately following the total RNA prep so that both the RNA and protein derive from the same sample. After the sample has been

applied to the Direct-Zol column, collect the flow-through containing the protein. Protein can then be prepared from the flow-through in accordance with the protocol described by trizol reagents. Once a protein pellet has been obtained, it is solubilized using a 1:1 ratio of 8M urea and 1% SDS. The protein is now ready for analysis by western blot. Proteins were separated on SDS-PAGE gels ranging for 6-10% and western blotted. Proteins of interest were probed with antibodies in accordance with manufacturer protocols. Specifically, primary antibodies were used at 1:2000 dilution while α -rabbit secondary antibody was used at 1:5000 for recognition of all primary antibodies.

Luciferase Reporter Construction

In order to assay the requirement of the 3' UTR of target genes to mediate regulation by Pumilio proteins, a dual luciferase assay was used. The system used was adapted from a methods paper entitled "A guide to design and optimization of reporter assays for 3' untranslated region mediated regulation of mammalian messenger RNAs" (Van Etten *et al.*, 2013). Two vectors were purchased from Promega to perform these assays. The psiCHECK-1 vector contains the renilla luciferase gene driven by a SV40 enhancer/promoter. Immediately following the coding sequence of renilla is a multiple cloning site (MCS) for inserting 3' UTRs of genes to be experimentally tested. Utilizing the NotI and XhoI sites in the MCS, I designed primers for amplifying and cloning the 3' UTR of SNCA, LRRK2 (short version), LRRK2 (long version), SAT1, E2F3 (positive control) and E2F1 (negative control). Two variants of the 3' UTR of LRRK2 were described on Pubmed (full length 3' UTR and a shorter 3' UTR), thus I cloned both of them for further

analysis. Primers used for amplification and cloning can be found in Table 2.4. Amplification of 3' UTRs was done using cDNA from SH-SY5Y cells. Basic cloning techniques were used and the plasmids were sequenced following construction. For mutations in Pumilio binding sites and other experimental mutations, Stratagene QuikChange XL Site-Directed Mutagenesis was used. Primers used for mutations created in 3' UTRs of target genes can be found in Table 2.4.

Transfection of Reporter Constructs

24 hours post knockdown application, I transfected the reporter constructs. To transfect reporter constructs, Polyjet transfection agent was used. This is an optimized transfection reagent for DNA. Polyjet works by forming a reagent/DNA complex utilizing a 3:1 volume to weight ratio. For example, if using 100ng of plasmid then 0.3 μ L of Polyjet is required. For data presented herein, 25ng of the internal reporter (firefly) was transfected with 75ng of the experimental reporters (renilla). The total weight of DNA was 100ng per well, thus 0.3 μ L of Polyjet was required per well. Cocktails of reporter transfections were made whenever possible. Reporters were kept at 50ng/ μ L for ease of use. The following describes how to apply the reporters to 10,000 cells in a 96 well format. A cocktail must be made for both the DNA plus growth medium (- antibiotics) and Polyjet plus growth medium (-antibiotics). For 10 wells where 10 μ L of transfection complex plus 90 μ L of fresh media (-antibiotics) is added to the well, two separate cocktails are made. One cocktail contains 5 μ L of firefly DNA (125ng total), 15 μ L of experimental renilla reporter DNA(375ng total) and 30 μ L of media (-antibiotics). Next, a second cocktail contains 3 μ L of Polyjet with 47 μ L of media. The two cocktails are then

combined mixing the polyjet into the DNA (order is important, do not mix the reverse way) and allowed to complex for 10-15 minutes. During this time remove the media from the cells and add back 90 μ L of fresh media (-antibiotics), allowing 10 μ L of space for the transfection complex. After 10-15 minutes, add 10 μ L of the transfection complex to each well and mix by gentle pipetting. There is now the correct final volume of 100 μ L per well. Allow to sit 24-48 hours before assaying reporters by the Dual-Glo assay.

Dual-Glo Luciferase Assay

In order to measure luciferase, white cell culture plates must be used. Also, all edge wells should be filled with medium to reduce background and misreading during data acquisition on a luminometer. Do not use the edge wells for experiments as they tend to evaporate and cause issues with luminescence. The Dual-Glo luciferase assay was performed using the manufactures protocol. In brief, an equal volume of Dual-Glo reagent (100 μ L) was added to each well and pipetted to mix. The mixture was allowed to sit at least 10 minutes and firefly luminescence was measured using the Victor 2 plate reader. Alternatively, the nano drop could be used; however, I did not use it and do not have the appropriate protocol. After the control firefly readings have been acquired, add a volume of Dual-Glo Stop and Glo reagent equal to the original well volume (100 μ L) and mix. Again, allow to sit at least 10 minutes and then assay using the Victor 2 plate reader. Background readings of the media alone are important for normalization as well as analysis of a control reporter (no added 3' UTR) on each plate. All normalization and presentation of data was done as described (Van Etten *et al.*, 2013).

Overexpression of Pumilio Proteins

Overexpression of Pum1 and Pum2 or empty vector was accomplished using Polyjet transfection reagent. Transfection of Pumilio expression constructs with reporter constructs was done in the same manner as previously described. In order to get to 100ng total DNA weight, the internal reporter and experimental reporter were dropped down to 10ng and 30ng, respectively. The remaining 60ng was occupied by Pum expression vectors (a kind gift from the Dyson Lab (Miles *et al.*, 2012)) or empty vector controls. Luciferase assays were done 24-48 hours post reporter/Pumilio overexpression transfection.

Overexpression of miRNAs

Overexpression of miRNAs was done in the same manner as the siRNA knockdowns. In brief, the treatment was done the day after plating 10,000 cells as was done with siRNA knockdowns. If knockdown and miRNA overexpression are to be done together, simply use the same concentration of siRNA/miRNA (5nM final) in a cocktail as previously described. 24 hours after siRNA or miRNA transfection, transfect the reporter constructs and perform luciferase assays 24-48 hours later.

RESULTS

All of the results in this chapter were completed by me.

Knockdown of Pumilio proteins results in up regulation of genes involved in Parkinson's disease progression.

Examination of the 3' UTRs of Parkinson's associated genes SNCA, LRRK2 and SAT1 revealed that there are one or more putative PREs present in each (Figure 4.1). To begin to elucidate if these mRNAs are regulated by Pumilio proteins, I performed RNAi knockdown of both Pum1 and Pum2 by transfecting SH-SY5y cells with "SMARTpools" of

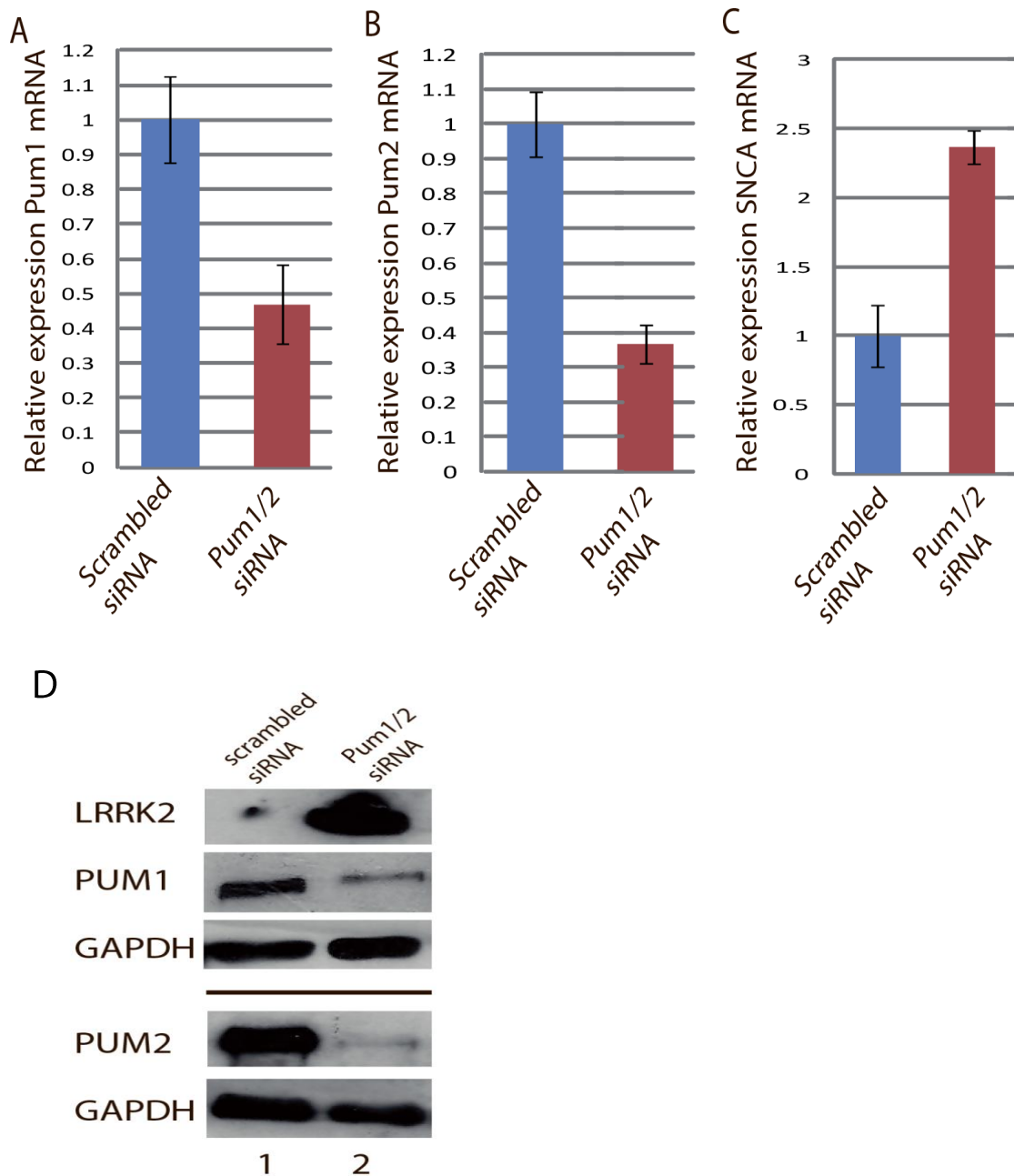


Figure 4.3. Knockdown of Pum1 and Pum2 results in up-regulation of SNCA mRNA and LRRK2 protein levels. Shown are quantitative PCR analyses of PUM1 (A), PUM2 (B) and SNCA (C) mRNA levels extracted from SH-SY5Y cells transfected with either scrambled siRNAs (blue) or siRNA SMARTpools against both Pum1 and Pum2 (red). Expression levels were normalized to GAPDH mRNA levels in each experimental set. Data are averages of biological triplicates performed in technical triplicate. (D) Shown are representative Western blot analyses of protein extracted from SH-SY5Y cells transfected with either scrambled siRNAs (lane1) or siRNA SMARTpools against both Pum1 and Pum2 (lane2). The top three panels derive from a single blot, while the bottom two panels derive from a separate blot, although still the same protein samples. Results were observed in triplicate.

small interfering RNAs (siRNAs) against both Pum1 and Pum2 sequences. Specifically, 10,000 cells were plated per well and treated with siRNAs in triplicate wells. Cells were allowed to sit for 48 hours to maximize protein knockdown. I chose to knockdown both Pufs simultaneously since Pum1 and Pum2 are thought to be partially redundant and I wanted to achieve the maximum effect in a single experiment. Cells were alternatively transfected with scrambled siRNAs as a negative control. Following knockdown, total RNA and protein were prepared from each well. 500ng of total RNA from each well was then subjected to reverse transcription and cDNA was diluted for use in qRT-PCR. As shown in Figure 4.3 A & B, qRT-PCR analysis demonstrated ~60% reduction in Pum1 and Pum2 mRNA levels, with a corresponding 80-90% reduction in Pum1 and Pum2 proteins levels as measured by Western blot (Figure 4.3D). The larger reduction in protein levels is expected as siRNAs can affect both stability and translation of bound mRNAs. Having established successful knockdown of both Pum1 and Pum2, I performed qPCR analysis of SNCA mRNA, a key Parkinson's mRNA encoding α -synuclein and whose 3' UTR contains a perfect PRE (Figure 4.1). SNCA levels increased >2-fold in response to Pum1/Pum2 knockdown (Figure 4.3C). This data supports the hypothesis that the Puf proteins act to repress SNCA mRNA levels. I attempted to assay LRRK2 mRNA levels by qPCR; however, the two primer sets attempted did not work consistently. To determine if LRRK2 is a target of Pumilio regulation, I analyzed LRRK2 protein levels by Western blot using anti-LRRK2 antibody. The LRRK2 3' UTR contains three perfect PREs and five imperfect PREs (Figure 4.1). LRRK2 levels are barely detectable in control lanes when treated with scrambled siRNA (Figure 4.3D, lane 1); however, with Pum1/Pum2

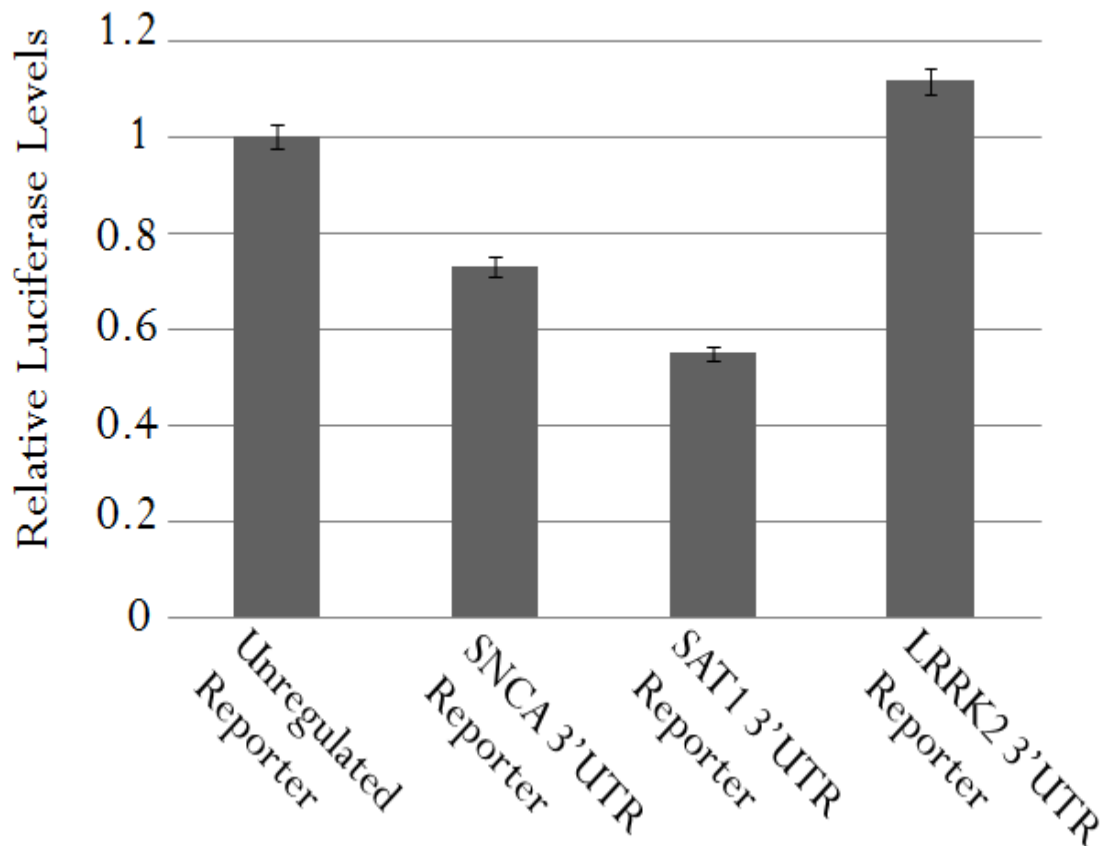


Figure 4.4. 3' UTRs of PD mRNAs are sufficient to confer regulation. Shown are relative luciferase levels of reporters containing either a minimal 3' UTR (Unregulated reporter) or 3' UTRs from PD mRNAs (SNCA, SAT1 or LRRK2). The 3' UTRs of both SNCA and SAT1 reduced luciferase levels compared to the unregulated reporter. The 3' UTR of LRRK2 increased luciferase levels compared to the unregulated reporter.

knockdown, LRRK2 levels significantly increased (Figure 4.3D, lane 2). This data further supports the role of Puf proteins in repressing levels of Parkinson's associated proteins. I was not able to successfully assay SAT1 mRNA or protein due to technical challenges.

Pumilio Proteins regulate Parkinson's disease associated genes through their 3' UTRS.

I hypothesized that SNCA, LRRK2 and SAT1 are targeted for repression because the Pumilio proteins are binding to recognition sequences in the 3' UTRs, as opposed to nonspecific effects or because regulation of the PD genes is downstream of a direct Pumilio target. To test the role of Pumilio binding to the 3' UTR for regulation of PD genes, I first examined the sufficiency of the 3' UTRs to confer Pumilio regulation. Specifically, the commercially available Renilla Luciferase reporter plasmid pGL4 (Promega) was used in which I cloned the 3' UTRs of either SNCA, SAT1 and LRRK2 downstream of the luciferase coding region. The constructed Renilla Luciferase/3' UTR plasmids were transfected into HEK293 cells, along with a Firefly Luciferase reporter plasmid to control for transfection. The transfection and subsequent luciferase reporter analysis/quantification were performed according to established protocols (Van Etten *et al.*, 2013). Both the SNCA and SAT1 3' UTR vectors resulted in repressed expression as compared to the unregulated Renilla Luciferase reporter lacking a 3' UTR insertion, indicating that the SNCA and SAT1 3' UTRs contain repressive cis- elements (Figure 4.4). Expression of the LRRK2 3' UTR vector was similar to the unregulated control; however, LRRK2 may have both positive and negative cis-elements that balance expression (Figure 4.4).

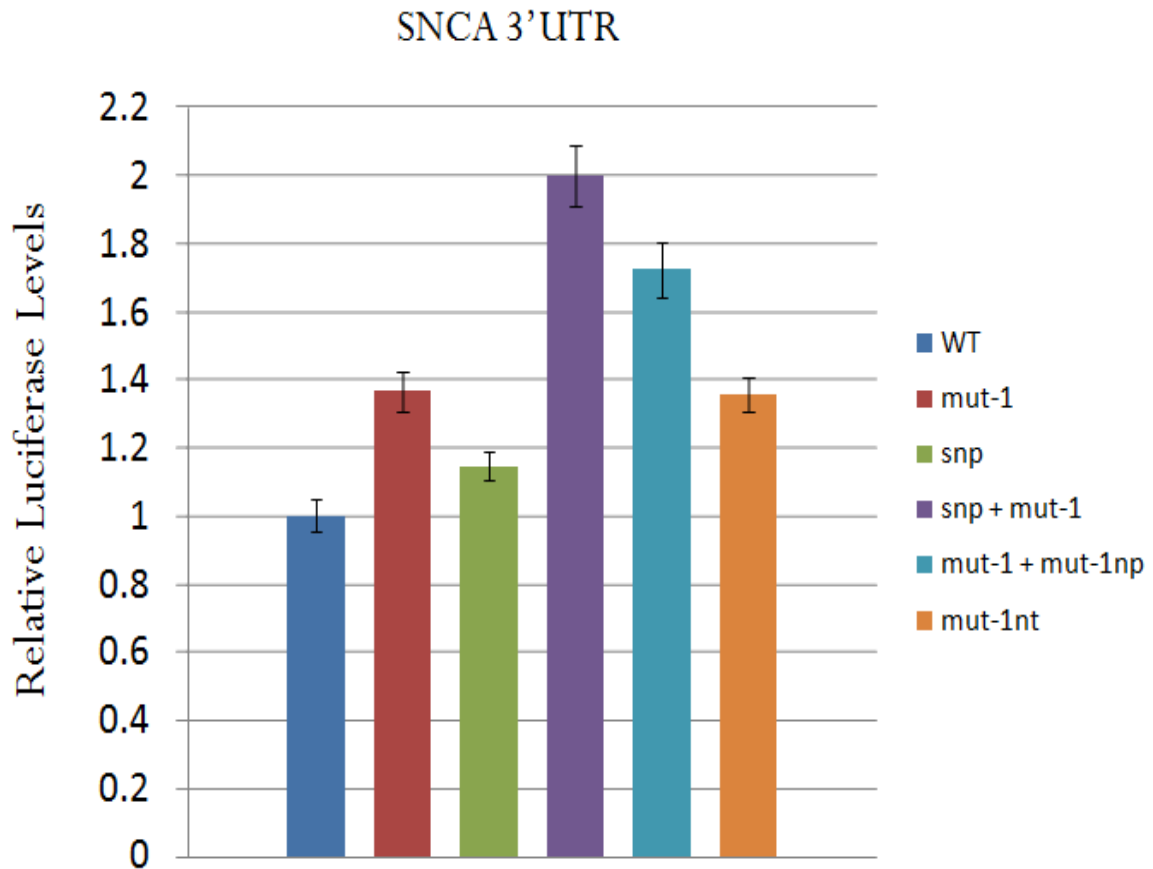


Figure 4.5. Mutation of PREs in the SNCA 3' UTR increases reporter luciferase levels.

Wild type (WT) luciferase levels were normalized to 1. Shown are various mutations in the 3' UTR of SNCA. mut-1 represents a mutation of the first canonical PRE (UGUA to ACAC), SNP represents a previously described mutation affecting SNCA luciferase level (Sotiriou *et al.*, 2009), mut-1 + mut-1np represents a mutation to the canonical site as well as a mutation to the most similar non-perfect site, mut-1nt represents a 1 nucleotide mutation of the canonical PRE (UGUA to UCUA).

To further validate the 3' UTRs of SNCA, LRRK2 and SAT1 as bona fide targets of Pumilio regulation, I mutated putative PREs. If a PRE is functioning in a repressive manner, I would observe an increase in luciferase levels coming from the mutant construct when compared to wild-type. Using site-directed mutagenesis, several mutations were made in the SNCA 3' UTR. First, mutation of the first canonical PRE was made by changing UGUUAUAUA to ACACUAUA (mut-1). Mutation of the core UGUA has been previously shown to be sufficient to abolish Pumilio mediated regulation (Miller and Olivas, 2011). Indeed, upon mutation of the PRE an increase in luciferase levels was observed (Figure 4.5). Furthermore, mutation of the SNCA 3' UTR to create a relevant single nucleotide polymorphism (snp) known to be involved in Parkinson's disease also increased luciferase levels and served as a positive control for my assay (Figure 4.5) (Sotiriou *et al.*, 2009). The effect of the SNP and mut-1 were additive, as the largest increase in luciferase levels was observed in this double mutant. Interestingly, mutation of a non-canonical putative PRE in addition to the canonical PRE increased luciferase levels even further than the single PRE mutation (mut-1 + mut-1np(non-perfect)). Finally, I mutated one nucleotide (mut-1nt) of the canonical PRE (UGUA to UCUA) and observed an identical increase in luciferase level to that of the tri-nucleotide mutation of mut-1. Thus, both the canonical PRE and the non-canonical PRE located in the 3' UTR of SNCA are bona fide cis-regulatory elements likely regulated by Pumilio proteins. Additionally, the SNP combined with mut-1 had the largest effect, suggesting these cis elements may be synergistic. Similar to mutational analysis of the SNCA 3' UTR, I mutated the LRRK2 3' UTR and the SAT1 3' UTR. Mutation of the first canonical PRE in

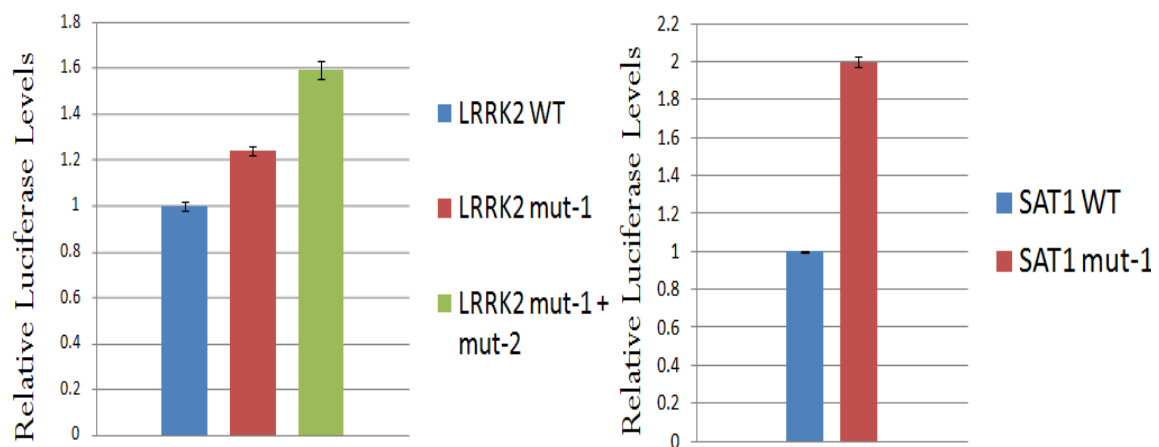


Figure 4.6. Mutation of PREs in the LRRK2 and SAT1 3' UTRs increases reporter levels. Wild type (WT) luciferase levels were normalized to 1. Shown are various mutations in the 3' UTR of LRRK2 and SAT1. LRRK2 mut-1 represents a mutation of the first canonical PRE (UGUA to ACAA), mut-1 + mut-2 represents mutation to the first two canonical PREs in the LRRK2 3' UTR. SAT1 WT represents the wild type 3' UTR, SAT1 mut-1 represents mutation of the PD relevant, non-canonical PRE (UGUA to ACAA).

the LRRK2 3' UTR was made as well as a double mutant consisting of mutations to the first two canonical PREs. Mutation of the first PRE increased luciferase levels compared to wild-type, and the double mutation of the first two PREs increased luciferase levels even further, suggesting that the first two PREs contribute to Pumilio-mediated regulation of LRRK2 (Figure 4.6). Additional analysis of the remaining canonical PREs as well as non-canonical PREs is needed to determine the full extent of Puf regulation. Furthermore, mutation of the non-canonical, clinically relevant (Lewandowski *et al.*, 2010) PRE in the SAT1 3' UTR increased luciferase levels compared to wild-type, indicating that Pumilio functions through this site (Figure 4.6). These results further support the hypothesis that Puf proteins are regulating SNCA, LRRK2 and SAT1 directly, through interactions with their 3' UTR.

Pumilio overexpression reduces reporter luciferase levels

To further validate SNCA, LRRK2 and SAT1 as bona fide targets of Pumilio regulation, I obtained Pum 1 and Pum2 overexpression plasmids (14) and co-expressed both vectors in HEK-293 cells with the Renilla and Firefly Luciferase reporters. Overexpression of Pum1 and Pum2 significantly reduced expression of all three 3' UTR reporters, suggesting that SNCA, SAT1 and LRRK2 are all directly responsive to Puf regulation (Figure 4.7). Overexpression of the empty vector had no effect on reporter expression (data not shown). Similar results have been seen previously for the Puf targeted E2F3 3' UTR in response to Pum1/2 overexpression (14). We also tested whether co-overexpression of Nanos affected the degree of Puf repression, since Nanos often works as a complex with Pufs to regulate target mRNAs and its levels may be

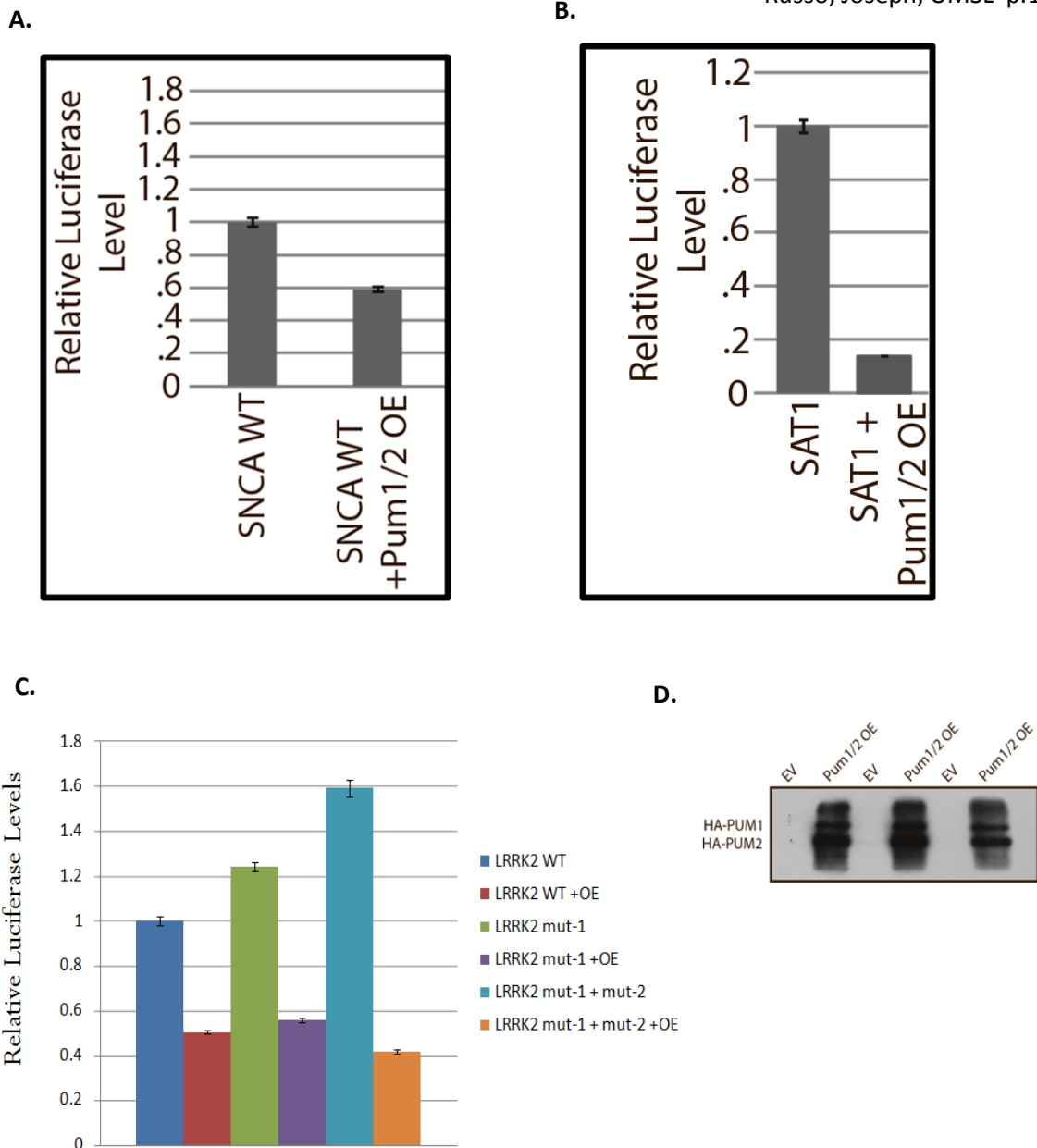


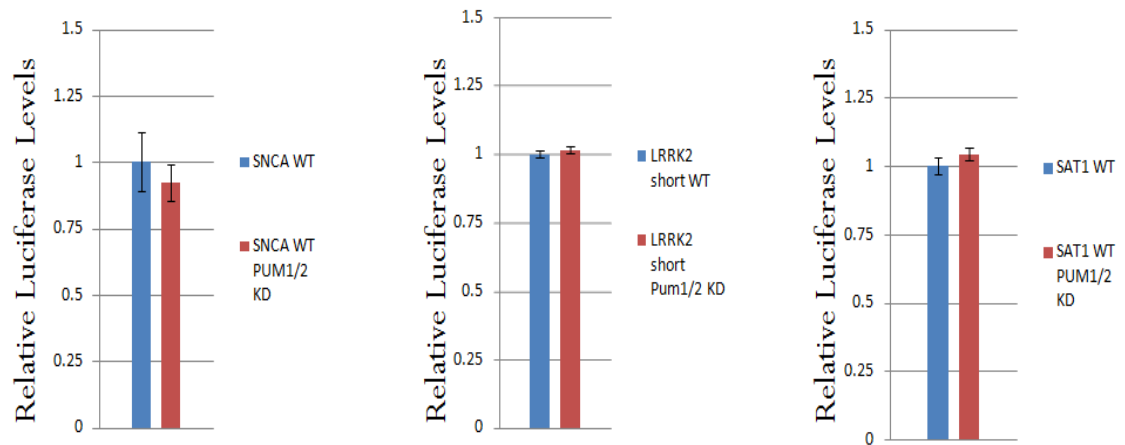
Figure 4.7. Overexpression of Pum1/Pum2 decreases Luciferase levels of reporters containing SNCA, SAT1 and LRRK2 3' UTRs. (A) Shown are relative Luciferase levels of the SNCA reporter during empty vector (SNCA WT) or during Pum1/2 overexpression (OE). (B) Shown are relative Luciferase levels of the SAT1 reporter during empty vector (SAT1) or during Pum1/2 overexpression (OE). (C) Shown is relative Luciferase levels of the LRRK2 reporter and mutant reporters during empty vector or Pum1/2 overexpression (OE). (D) Western blot displaying HA-Pum1/2 overexpression. The middle band is HA-Pum1 and the lowest band is HA-Pum2.

limiting in the cells. However, we did not see any differences in Puf-mediated repression with or without co-overexpression of Nanos (data not shown). When evaluating the mutant reporter constructs, we also observed a decrease in luciferase levels upon Pum1/2 overexpression. Specifically, with the LRRK2 mutant 3' UTRs (mut-1, mut-1+mut-2) luciferase levels were reduced similar to that of wild-type (Figure 4.7). Also, overexpression of Pum1 and Pum2 on the control reporter (no added 3' UTR) caused a decrease in luciferase levels (data not shown) suggesting that although overexpression reduces luciferase levels of reporters, it may be due to non-specific effects. Alternatively, the non-specific effects may be due to the high level of Pum overexpression and titration may be needed to limit off-target effects. Regarding the LRRK2 mutant 3' UTRs, it may be that the additional canonical and non-canonical PREs are used during overexpression such that the effect of the mutations are masked. Together, this reporter data suggests that Puf regulation of the SNCA, SAT1 and LRRK2 mRNAs is directly acting through the 3' UTRs, but more experiments are needed to evaluate the overexpression effects.

Knockdown of Pumilio proteins in HEK-293 cells does not affect reporter luciferase levels

To further support the hypothesis that Pumilio proteins act directly on SNCA, LRRK2 and SAT1 through interactions with the 3' UTR, I performed Pumilio knockdown studies in the HEK-293 cells and evaluated wild-type reporter responses. If Pumilio proteins act through the 3' UTR of these mRNAs we would expect an increase in luciferase levels upon knockdown of Pumilio proteins. Knockdown of Pum1 and Pum2 did not increase luciferase levels of the reporters (Figure 4.8A). These

A.



B.

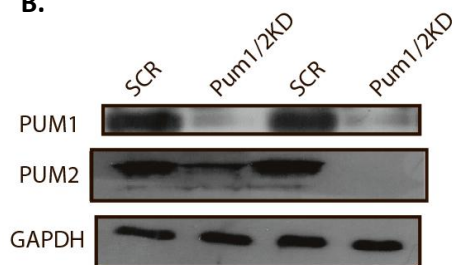


Figure 4.8. Knockdown of Pum1/2 has no effect on reporters bearing 3' UTRs of SNCA, LRRK2 or SAT1. (A) Shown are bar graphs representing relative luciferase levels during scrambled siRNA treatment (SNCA WT, LRRK2 short WT, SAT1 WT) or upon knockdown of Pum1/2 (PUM1/2 KD). (B) Shown is a representative western blot of Pum1/2 knockdown during experiments.

results were not because of failure to knockdown Pumilio proteins (Figure 4.8B). Given the fact that the 3' UTRs of target mRNAs were cloned from SH-SY5Y cells, it is possible that the response to knockdown I observed on endogenous targets cannot be recapitulated with the reporters. It is of value to perform this assay in the SH-SY5Y cells as varying expression levels of Pumilio proteins (increased levels in SH-SY5Y cells) is present in the two cell lines as observed by Western blot.

DISCUSSION

Many genes are implicated in the progression of PD, and much research has focused on mutations of genes found in PD patients and how they contribute to disease progression. Recent research has begun to evaluate the critical role of aberrant expression of PD genes and its contribution to disease progression; however, an area of research lacking in the field is the role of post-transcriptional gene regulation of PD genes. Specifically, there is sparse research on the role of RNA binding proteins (RBPs), which include the Puf family, and how they may contribute to altered expression of PD genes containing no apparent mutations. By elucidating the role of Puf proteins on post-transcriptional control of PD genes, we will provide a novel avenue for therapeutic approaches to PD. We will also make significant strides in understanding Puf function in human cells and offer new targets of Puf mediated regulation in humans for future studies. Until recently, the understanding of Puf regulation in humans has remained elusive. Strides have been made implicating the dependency of miRNA mechanisms on Puf function, yet experimental analyses of Puf targets and regulatory mechanisms are few. In this work, I identify 3 mRNAs, SNCA, LRRK2 and SAT1 as targets of Pumilio

regulation in human cell lines. This discovery provides evidence of post-transcriptional control contributing to the regulation of genes involved in PD. Furthermore, these studies implicate Pumilio proteins in the progression of PD. Further research will determine the interplay between the miRNA system and Pumilio proteins through evaluation of various mutant 3' UTRs of the reporter constructs as well as structural analysis of target 3' UTRs using fluorescent resonance energy transfer (FRET). There will be challenges in interpreting the data from 3'UTR mutational studies as a tri-nucleotide mutation may disrupt secondary structure and indirectly aid miRNA function. Thus, it was important to establish that a single nucleotide change is sufficient to disrupt Pumilio function. It is more appropriate to perform single mutations as to maintain secondary structure as much as possible. Maintaining secondary structure will be critical in understanding the interplay between the miRNA system and the Pumilio system. These studies will further our understanding of Pumilio and miRNA interdependency for regulating target mRNAs involved in PD progression.

Table 1.4. siRNAs, miRNAs and antibodies used in this study.

Product	Sequence/Target	Source
Silencer Pre-designed siRNA-PUM1	gcuucgguauauagguccutt	Ambion (4392420)
Silencer Select Pre-designed siRNA-PUM2	gcaacuaauucagcuaauctt	Ambion(AM16708)
Accell SMARTpool siRNA PUM1	gcuucaguuugugacauua cuuucagggugguuguuu ucaucuagucucaaguaua gccaugucuugaagugugu	Thermo Scientific (E-014179-00-0005) (9698)
Accell SMARTpool siRNA PUM2	gccgauugcaaagauuuua gcacuaaucugcaaucuaa gcaugguucuagauucaua guguauaauuaaaucguu	Thermo Scientific (E-014031-00-0005) (23369)
Accell Human Control siRNA kit	N/A	Thermo Scientific (K-005000-R1-01)
hsa-miR-153-3p	uugcauagucacaaaagugauc	Ambion(MC10122)
hsa-miR-205-5p	uccuucuuuccaccggagucug	Ambion(MC11015)
hsa-miR-410-3p	aaauaaacacagauggccugu	Ambion(MC11119)
hsa-miR—7-5p	uggaagacuagugauuuuguugu	Ambion(MC10047)
Anti-GAPDH(in rabbit)	Human GAPDH	Abcam (EPR6256)
Anti-Pumilio1(in rabbit)	Human/mouse Pum1	Abcam (EPR3795)
Anti-Pumilio2(in rabbit)	Human/mouse Pum2	Abcam (EPR3813)
Anti-Synuclein	Human/mouse/rat SNCA	Abcam
Anti-LRRK2	Human/mouse/rat LRRK2	Abcam

Table 2.4. Oligos used in this chapter

oWO	Sequence	Use
614	<i>tgcattggtgtggcaacagt</i>	qPCR SNCA up primer
615	<i>tccactgtcttctgggctactg</i>	qPCR SNCA down primer
616	<i>agttcatccatggaacgcacctg</i>	qPCR PUM1 up primer
617	<i>agcaaacatcgatggcctactgg</i>	qPCR PUM2 up primer
620	<i>ttgggtgctgctataggctcag</i>	qPCR PUM2 down primer
621	<i>acttgagaactgccaactgatg</i>	qPCR GAPDH up primer
622	<i>tctcttctctgtgtcttctgta</i>	qPCR GAPDH down primer
623	<i>agctcatttctgtgtatgacaacg</i>	qPCR LRRK2 up primer
707	<i>tgatgagaagcaacgcaagcc</i>	qPCR LRRK2 down primer
708	<i>ggaaccctcgcttattcaggagtc</i>	SNCA 3'UTR clone up(xhoI)
758	<i>ccagctcgagaaatatcttctgctccagtttcttgag</i>	SNCA 3'UTR clone dn(notI)
759	<i>ccaggcgccgcataatcttctgcaatgagataacgtttta</i>	LRRK2 3'UTR clone up(xhoI)
760	<i>gcatctcgagagagaaataggaattgtctttgg</i>	LRRK2 short clone dn (notI)
761	<i>gcatgcgccgcttcacataccaacaaaggac</i>	LRRK2 full clone dn (notI)
762	<i>gcatgcgccgcttattatttccatttaaatatggtattc</i>	SAT1 3'UTR clone up(xhoI)
763	<i>gcatctcgagggagtgctgctgtagatgacaacc</i>	SAT1 3'UTR clone dn (notI)
764	<i>gcatgcgccgcaagagtagtttataaaaaagaatcaaacag</i>	E2F1 3'UTR clone up(xhoI)
765	<i>gcatctcgagcagggcttgaggagacca</i>	E2F1 3'UTR clone dn (notI)
766	<i>gcatgcgccgctggatctgcttttgagttagga</i>	E2F3 3'UTR clone up (xhoI)
767	<i>gcatctcgagttatgcttcgtgtgaactctc</i>	E2F3 3'UTR clone dn (notI)
768	<i>gcatgcgccgcaaatcacccattgaagtggtaag</i>	qPCR firefly up primer
769	<i>gatcctcaacgtgcaaaagaagc</i>	qPCR firefly down primer
770	<i>tcacgaaggtgtacatgctttgg</i>	qPCR renilla up primer
771	<i>cgcaactacaacgcctaccttc</i>	qPCR renilla down primer
772	<i>ccctcgacaatagcgttggaata</i>	psiCHECK sequence primer
773	<i>gacgctccagatgaaatgggtaag</i>	psiCHECK rev sequence primer
774	<i>cgcaatttccccgaaaagt</i>	LRRK2 sequence primer
775	<i>ctaggaaagacacagaaactctc</i>	E2F1 sequence primer
776	<i>gattgaagctttaatggagcg</i>	E2F3 sequence primer #1
777	<i>cagccttctggatgaagaacctg</i>	E2F3 sequence primer #2
778	<i>ggctctcttacaccgcactc</i>	E2F3 sequence primer #3
779	<i>ccaggtagatttccacaatatg</i>	E2F3 sequence primer #4
780	<i>ggtgtacattaattagatgtcc</i>	SNCA SDM up primer perfect site
798	<i>gcgatgtgttttattcactttgtgttacctataaatggtgagaattaaaaataaa acg</i>	SNCA SDM dn primer perfect site
799	<i>cgttttatttttaattctcaccatttatagtgttaacacaaagtgaataaaacacatc gc</i>	SNCA SDM up primer snp
804	<i>aaatactaaatatgaaattttaccattttgtgatgtgttttattcactgtgtttgta t</i>	SNCA SDM dn primer snp
805	<i>atacaaacacaagtgaataaaacacatcacaaaatggtaaaatttcatattta gtattt</i>	SNCA SDM up perfect site w/snp
806	<i>aatactaaatatgaaattttaccattttgtgatgtgttttattcactgtgtttacac</i>	SNCA SDM dn perfect site w/snp
807	<i>gtgttaacacaagtgaataaaacacatcacaaaatggtaaaatttcatatttag tatt</i>	SNCA SDM up 1 st nonperfect site
808	<i>agatgttccatccacaccaagtgtctcagtt</i>	SNCA SDM dn 1 st nonperfect site

809	<i>aactgagcacttggtgtggatggaacatct</i>	SNCA SDM up 2 nd nonperfect site
810	<i>atttctcaaagtttttacagacactctcgaagtcttccatcag</i>	SNCA SDM dn 2 nd nonperfect site
811	<i>ctgatggaagacttcgagagtgtctgtataaaactttgagaaat</i>	SNCA SDM up 3 rd nonperfect site
812	<i>gtgattgaagtatcacaccctgccccact</i>	SNCA SDM dn 3 rd nonperfect site
813	<i>agtgggggcaggggtgtgatacttcaatcac</i>	LRRK2 SDM up 1 st site
814	<i>gataggaaaattattctctcctctacacaatattttttaaaaatgttcacatgg aaag</i>	LRRK2 SDM dn 1 st site
815	<i>ctttccatgtgaacatttttaaaataaatattgtgtagaggagagaataattttc ctatc</i>	LRRK2 SDM up 2 nd site
816	<i>ggaatgttattatttttaatttaaatataacacaaaatacttaccagtaaattgtg attt</i>	LRRK2 SDM dn 2 nd site
817	<i>aaatacacatttactggttaagtattttgtgttatatttaaaaaataataa catcc</i>	LRRK2 SDM up 3 rd site
818	<i>acacaaaatacttaccagtaaattgacacttttaagaactatttaaaacacaat g</i>	LRRK2 SDM dn 3 rd site
819	<i>cattgtgttttaaatagttctttaaaagtgtcatttactggttaagtattttgtg</i>	LRRK2 SDM up 4 th site
820	<i>atcgaaatgcactatcatatatgtcacacaatattcaaatgaattgcactaata aagtc</i>	LRRK2 SDM dn 4 th site
821	<i>gactttatttagtgcaaattcatttgaatattgtgtagcatatatgatagtgcattt cgat</i>	SAT1 SDM up 1 st site
822	<i>gagggagtgtgcacacgatgacaacctcca</i>	SAT1 SDM dn 1 st site
823	<i>tggagggtgtcatcgtgtgcagcactccctc</i>	SAT1 SDM up 2 nd site
824	<i>cccaacttctcttgctttctatgtgttacacgtgaaataatagaatgagcaccca ttcc</i>	SAT1 SDM dn 2 nd site
825	<i>ggaatgggtgctcattctattatttcacgtgtaacagcatagaaagcaagaga agttggg</i>	SAT1 SDM up 3 rd site
826	<i>cttcattctcgtgagtcatttaaatgacaccaatgtacacactggtacttagag</i>	SAT1 SDM dn 3 rd site
827	<i>ctctaagtaccagtgtgtacattgggtgcatttaaatgactcacgagaatgaag</i>	SNCA SDM up 1NT
828	<i>gcgatgtgttttattcactttgtgtttctatataaatggtgagaattaaaataaaa cg</i>	SNCA SDM dn 1NT
829	<i>cgttttattttaattctcaccatttatatagaacacaaaagtgaataaaacacat cgc</i>	qPCR PUM2 up primer

Table 3.4. Plasmids Used in This Study

pWO	Description	Source
203	firefly luciferase reporter	Promega
204	renilla luciferase reporter	Promega
205	HA-Pum1 expression vector	Dyson Lab (16)
206	HA-Pum2 expression vector	Dyson Lab (16)
207	renilla-SNCA 3'UTR	Joe Russo
208	renilla-LRRK2 3'UTR (long)	Joe Russo
209	renilla-LRRK2 3'UTR (short)	Joe Russo
210	renilla-SAT1 3'UTR	Joe Russo
211	renilla-E2F1 3'UTR	Joe Russo
212	renilla-E2F3 3'UTR	Joe Russo
213	renilla-SNCA 3'UTR mut-1	Joe Russo
214	renilla-SNCA 3'UTR snp	Joe Russo
215	renilla-SNCA 3'UTR mut-1, snp	Joe Russo
216	renilla-SNCA 3'UTR mut-1, mut-1 st non-perfect site (nps)	Joe Russo
217	renilla-SNCA 3'UTR mut-1(1 nt), mut-1 st non-perfect site	Joe Russo
218	renilla-LRRK2 3'UTR mut-1	Joe Russo
219	renilla-LRRK2 3'UTR mut-1, mut-2	Joe Russo
220	renilla-SAT1 3'UTR mut-1 st non-perfect site(ref 20)	Joe Russo
221	renilla-SAT1 3'UTR mut-2 nd non-perfect site	Joe Russo
222	renilla-SAT1 3'UTR mut-1 st nps, mut-2 nd nps	Joe Russo
223	renilla-SNCA 3'UTR mut-1(1nt change at site)	Joe Russo

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CHAPTER 5: DISCUSSION

Conditional regulation of Puf proteins in yeast

Work on Puf proteins over the last decade has revealed a complicated system of mRNA regulation and protein surveillance. The Puf family of RNA binding proteins bind target mRNAs and regulate their turnover through interactions with cis-regulatory elements located in the 3' UTR of mRNAs. Early research revealed the importance of the core UGU element for binding of Puf proteins to target mRNAs and this recognition mechanism is conserved throughout eukaryotes (Gerber *et al.*, 2004; Bernstein *et al.*, 2005; Gerber *et al.*, 2006; Galgano *et al.*, 2008; Miller and Olivas, 2011; Campbell *et al.*, 2012). Recent research has shown evidence for flexibility of Puf proteins for binding target mRNAs with a wide variety of sequences downstream of the UGU (Valley *et al.*, 2012) (This work, Figure 3.1). Furthermore, mechanistic details of Puf protein function have continued to be elucidated revealing roles in mRNA decay and translational repression (Goldstrohm *et al.*, 2006; Goldstrohm *et al.*, 2008; Miller and Olivas, 2011; Blewett and Goldstrohm, 2012; Van Etten *et al.*, 2012; Weidmann and Goldstrohm, 2012; Hrit *et al.*, 2014). Initially, the Puf repeat domain was shown to be both necessary and sufficient for binding and regulating target mRNAs; however, more recent advances in the field suggest autonomous repression capabilities outside of the repeat domain as well (Weidmann and Goldstrohm, 2012).

The work presented herein furthers the field of Puf research by identifying an activation switch in response to environmental stress. Specifically, yeast Puf1p, Puf3p, Puf4p and Puf5p regulate target mRNAs based on the available carbon source in the

growth media. During fermentation (dextrose conditions) Puf proteins are active to stimulate rapid decay of target mRNAs; however, during respiration (galactose, ethanol conditions) Puf proteins are inactive and target mRNAs are derepressed. I show that Puf protein activity is not regulated by either altered expression or localization of the Puf proteins. Instead, I show several modes of activation specific for individual Puf proteins in yeast. One hypothesis of how Puf proteins may be inactivated is to eliminate RNA binding capacity in inactive conditions as has been observed with human Pum1 (Kedde *et al.*, 2010). Puf1p, Puf3p and Puf4p are all able to bind target mRNAs regardless of the carbon source; however, Puf5p binds target mRNAs less efficiently in respiration conditions. Thus, Puf5p likely alters its binding capacity to limit its regulatory effects on target mRNAs. Further support for this hypothesis comes from the identification of a mutant Puf5p which contains an aspartic acid mutation directly next to a known phosphorylated residue in Puf5p. This charge mimic greatly increased Puf5p repressive capacity compared to wild-type and this may be due to increased and/or more efficient binding to target mRNAs (this work).

A second hypothesis for the activation switch for Puf proteins is to alter interactions with known decay machinery during inactivating conditions. It is possible that critical components of the decay machinery are no longer bound to Puf proteins during stress, a condition which leads to stabilization of target mRNAs. Indeed, interactions between Puf3p and Pop2p are perturbed in galactose conditions, providing a possible mechanism for inactivation of Puf3p. In fact, Puf3p binding to a smaller version of Pop2p was increased in galactose conditions. Additionally, this smaller

version of Pop2p nearly exclusively binds a mutant form of Puf3p that can bind but not repress target mRNAs. It is possible that binding to the smaller version of Pop2p may inhibit repression of target mRNAs by affecting the deadenylation step of decay, as preliminary data suggests this is the case. Previous research has identified a phosphorylation event of Thr97 of Pop2p by Yak1p in response to glucose deprivation (Moriya *et al.*, 2001). I sought to determine if this modification is responsible for disrupted binding between Puf3p and Pop2p, thereby affecting mRNA turnover. Transcriptional shut-offs in a *yak1Δ* revealed a constitutively active Puf3p in galactose conditions, further supporting the hypothesis that the phosphorylation of Thr97 on Pop2p is required for Puf3p inactivation.

Human Pumilio proteins regulate mRNAs involved in Parkinson's Disease

Much research on Puf proteins has focused on using lower eukaryotes such as *S. cerevisiae* and *Drosophila melanogaster*. Although large scale studies have been done to identify target mRNAs regulated by human Pumilio (Fox *et al.*, 2005; Galgano *et al.*, 2008), identification of bona fide targets is sparse (Kedde *et al.*, 2010; Miles *et al.*, 2012; Fernandez *et al.*, 2014). In addition, recent research shows that Pumilio repressive function may also include interplay with the miRNA system, although the mechanism remains elusive. Data supports a model in which Pumilio proteins bind target mRNAs and relieve secondary structure to free up miRNA seed regions that have otherwise been inaccessible (Kedde *et al.*, 2010). In addition, it has been shown that human Pumilio proteins have their own repressive capacity independent of the miRNA system (Van Etten *et al.*, 2012) and global miRNA regulation is not dependent on Pumilio

proteins as many miRNA targeted mRNAs do not contain PREs (Galgano *et al.*, 2008; Incarnato *et al.*, 2013; Jiang *et al.*, 2013). Thus, it is important to identify bona fide targets of Pumilio regulation in humans to begin to understand the multifaceted mechanism by which regulation occurs.

I have identified several key genes involved in the progression of PD as bona fide targets of Pumilio regulation. Bioinformatic analysis of 3' UTRs of genes involved in PD revealed that SNCA and LRRK2 contain putative PREs, and physical association studies revealed that some PD genes are bound to Pum1 (Galgano *et al.*, 2008). In addition, SAT1 contains no canonical PREs; however, it does contain two non-canonical PREs. In fact, the SAT1 PRE has been implicated in post mortem PD brains where a deletion of a UGU element was observed (Sotiriou *et al.*). My studies reveal that all three genes are likely bona fide targets of Pumilio regulation. Knockdown of Pum1 and Pum2 resulted in increased expression of both SNCA and LRRK2 at the mRNA and protein level, respectively. Furthermore, luciferase assays utilizing the 3' UTRs of SNCA, LRRK2, and SAT1 revealed that they are likely Pumilio targets since mutation of the PRE resulted in increased expression of a luciferase reporter. No other regulatory proteins have been shown to bind the specific PREs, further supporting these mRNAs as bona fide Pumilio targets. One issue that occurred in validating these targets is that when Pumilio proteins were knocked down in HEK-293 cells the reporters did not respond. Previous studies where knockdown showed a phenotype were done in SH-SY5Y cells; however, due to technical issues with transfection in SH-SY5Y cells, reporter based assays are

done in HEK-293 cells. This may be the source of inconsistency, as expression of Pumilio proteins are higher in SH-SY5Y cells compared to HEK-293 cells.

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